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(54) Title: FLAVONOIDS FOR CYSTIC FIBROSIS THERAPY (57) Abstract Compositions and methods for therapy of cystic fibrosis and other conditions are provided. The compositions comprise one or more compounds such as flavones and/or isoflavones capable of stimulating chloride transport in epithelial tissues. Therapeutic methods involve the administration (e.g., orally or via inhalation) of such compositions to a patient afflicted with cystic fibrosis and/or another condition responsive to stimulation of chloride transport.		

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FLAVONOIDS FOR CYSTIC FIBROSIS THERAPY

TECHNICAL FIELD

5 The present invention relates generally to the treatment of cystic fibrosis. The invention is more particularly related to compositions comprising one or more compounds such as flavones and/or isoflavones, which may be used to activate chloride transport (*i.e.*, absorption and/or secretion) in epithelial tissues of the airways, the intestine, the pancreas and other exocrine glands, and for cystic fibrosis therapy.

10

BACKGROUND OF THE INVENTION

 Cystic fibrosis is a lethal genetic disease afflicting approximately 30,000 individuals in the United States. Approximately 1 in 2500 Caucasians is born with the disease, making it the most common lethal, recessively inherited disease in that
15 population.

 Cystic fibrosis affects the secretory epithelia of a variety of tissues, altering the transport of water, salt and other solutes into and out of the blood stream. In particular, the ability of epithelial cells in the airways, pancreas and other tissues to transport chloride ions, and accompanying sodium and water, is severely reduced in
20 cystic fibrosis patients, resulting in respiratory, pancreatic and intestinal ailments. The principle clinical manifestation of cystic fibrosis is the resulting respiratory disease, characterized by airway obstruction due to the presence of a thick mucus that is difficult to clear from airway surfaces. This thickened airway liquid contributes to recurrent bacterial infections and progressively impaired respiration, eventually resulting in
25 death.

 In cystic fibrosis, defective chloride transport is generally due to a mutation in a chloride channel known as the cystic fibrosis transmembrane conductance regulator (CFTR; *see* Riordan et al., *Science* 245:1066-73, 1989). CFTR is a linear chloride channel found in the plasma membrane of certain epithelial cells, where it
30 regulates the flow of chloride ions in response to phosphorylation by a cyclic AMP-

dependent kinase. Many mutations of CFTR have been reported, the most common of which is a deletion of phenylalanine at position 508 ($\Delta F508$ -CFTR), which is present in approximately 70% of patients with cystic fibrosis. A glycine to aspartate substitution at position 551 (G551D-CFTR) occurs in approximately 1% of cystic fibrosis patients.

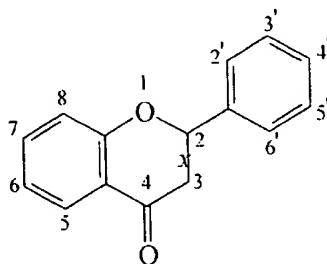
5 Current treatments for cystic fibrosis generally focus on controlling infection through antibiotic therapy and promoting mucus clearance by use of postural drainage and chest percussion. However, even with such treatments, frequent hospitalization is often required as the disease progresses. New therapies designed to increase chloride ion conductance in airway epithelial cells have been proposed, but
10 their long term beneficial effects have not been established and such therapies are not presently available to patients.

 Accordingly, improvements are needed in the treatment of cystic fibrosis. The present invention fulfills this need and further provides other related advantages.

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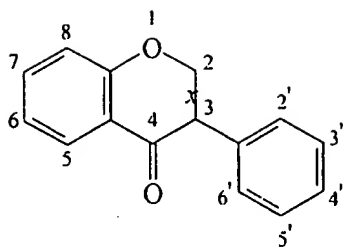
SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for enhancing chloride transport in epithelial cells and for the therapy of cystic fibrosis. Within one aspect, the present invention provides methods for enhancing chloride
20 transport in epithelial cells, comprising contacting epithelial cells with a compound selected from the group consisting of flavones and isoflavones, wherein the compound is capable of stimulating chloride transport and wherein the compound is not genistein. Within certain embodiments, the compound is (a) a polyphenolic compound having the general formula:



25

or



wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond; or (b) a stereoisomer or glycoside derivative of any of the foregoing polyphenolic compounds. Such compounds include, within certain embodiments, quercetin, apigenin, kaempferol, biochanin A, flavanone, flavone, dihydroxyflavone, trimethoxy-apigenin, apigenin 7-O-neohesperidoside, fisetin, rutin, daidzein and prunetin. For enhancing chloride transport in airway epithelial cells of a mammal, compounds may be administered orally or by inhalation. Other epithelial cells that may be employed include intestinal, pancreas, gallbladder, sweat duct, salivary gland and mammary epithelial cells. Within certain embodiments, the compound is combined with a substance that increases expression of a CFTR; and/or a chemical chaperone that increases trafficking of a CFTR to the plasma membrane.

Within other aspects, methods for enhancing chloride transport in epithelial cells may comprise contacting epithelial cells with a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid. Such compounds may further be used in combination with a flavone or isoflavone as provided above.

Within other aspects, the present invention provides methods for treating cystic fibrosis in a patient, comprising administering to a patient a compound as described above, wherein the compound is capable of stimulating chloride transport. Within certain embodiments, the compound is genistein, quercetin, apigenin, kaempferol, biochanin A, flavanone, flavone, dihydroxyflavone, trimethoxy-apigenin, apigenin 7-O-neohesperidoside, fisetin, rutin, daidzein or prunetin. Within other embodiments, the compound is resveratrol, ascorbic acid, ascorbate salts and

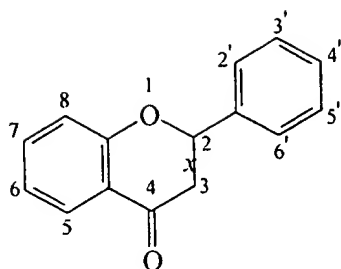
dehydroascorbic acid. Such compounds may be administered alone or in combination. Compounds may be administered orally or by inhalation. Within certain embodiments, the compound is combined with a substance that increases expression of a CFTR; and/or a chemical chaperone that increases trafficking of a CFTR to the plasma membrane.

5 Within further related aspects, the present invention provides methods for increasing chloride ion conductance in airway epithelial cells of a patient afflicted with cystic fibrosis, wherein the patient's CFTR protein has a deletion at position 508, the method comprising administering to a mammal one or more compounds as
10 described above, wherein the compound is capable of stimulating chloride secretion in the airway epithelial cells.

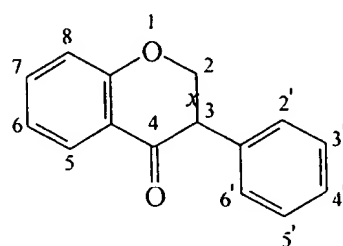
 Within still further related aspects, the present invention provides methods for increasing chloride ion conductance in airway epithelial cells of a patient afflicted with cystic fibrosis, wherein the patient's CFTR protein has a mutation at
15 position 551, the method comprising administering to a mammal one or more compounds as described above, wherein the compound is capable of stimulating chloride secretion in the airway epithelial cells.

 Within further aspects, pharmaceutical compositions for treatment of cystic fibrosis are provided, comprising (a) one or more flavones or isoflavones capable
20 of stimulating chloride transport and (b) one or more of: (i) a compound that increases expression of a CFTR in an epithelial cell; and/or (ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and; and in combination with a pharmaceutically acceptable carrier. Within certain embodiments, the flavone or isoflavone may be genistein, quercetin, apigenin, kaempferol, biochanin
25 A, flavanone, flavone, dihydroxyflavone, trimethoxy-apigenin, apigenin 7-O-neohesperidoside, fisetin, rutin, daidzein and/or prunetin, in combination with a pharmaceutically acceptable carrier.

 Within still further aspects, a pharmaceutical composition for treatment of cystic fibrosis may comprise: (a) a polyphenolic compound having the general
30 formula:



or



wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond; or a stereoisomer or glycoside derivative of any of the foregoing polyphenolic compounds; (b) a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid; and (c) a physiologically acceptable carrier.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a recording of transepithelial short-circuit current (Y axis) as a function of time (X axis), showing the effect of apigenin on the current across a Calu-3 cell monolayer. Measurements were performed in an Ussing chamber, where the basolateral membrane was permeabilized with α -toxin and a chloride gradient was applied across the apical membrane as a driving force. Tissue was first stimulated with cAMP (100 μ M). Apigenin (50 μ M) was subsequently added as indicated. The horizontal bar represents 100 seconds, and the vertical bar represents 12 μ A/cm².

Figure 2 is a recording showing the effect of quercetin on transepithelial short-circuit current across a Calu-3 cell monolayer in an Ussing chamber, where the basolateral membrane was permeabilized with α -toxin and a chloride gradient was applied across the apical membrane as a driving force. Tissue was first stimulated with cAMP (100 μ M). Quercetin (30 μ M) was subsequently added as indicated. Bars are 140 seconds (horizontal) and 12 μ A/cm² (vertical).

Figure 3 is a recording illustrating the dose-dependent stimulation of transepithelial chloride secretion by quercetin (in the amounts indicated) across a primary bovine tracheal epithelium. Amiloride (50 μ M) was added to block sodium transport as indicated. The CFTR channel blocker diphenylcarboxylate (DPC, 5 mM) was added as shown.

Figure 4 is a recording showing the effect of biochanin A on transepithelial short-circuit current across a Calu-3 cell monolayer in an Ussing chamber, where the basolateral membrane was permeabilized with α -toxin and a chloride gradient was applied across the apical membrane as a driving force. The tissue was first stimulated with forskolin (Fsk, 10 μ M). Subsequent addition of biochanin A (Bio, 100 and 300 μ M) was subsequently added as indicated.

Figure 5 is a cell-attached single channel patch clamp recording from a 3T3 cell expressing Δ F508-CFTR. The cell was treated with 10 μ M forskolin as shown. Genistein (50 μ M) and apigenin (50 μ M), were added where indicated by boxes. The holding potential was 75 mV, and channel openings were upward.

Figure 6 is a whole cell patch clamp recording on an airway epithelial cell homozygous for Δ F508-CFTR. Before the measurement, the cell was incubated for 2 days in 5 mM 4-phenylbutyrate. 30 μ M quercetin was added where indicated by the box. Further stimulation by forskolin (10 μ M) is also shown. The holding potential was -60 mV.

Figure 7 is a recording illustrating the effect of genistein on G551D-CFTR expressed in a *Xenopus* oocyte. Current was measured with the two-electrode voltage clamp technique. G551D-CFTR was injected in oocyte. Current was first stimulated with forskolin (10 μ M) and isobutylmethylxanthine (IBMX; 2 mM).

Genistein (50 μ M) was added as indicated. The right panel shows current voltage relations recorded after treatment with forskolin and IBMX (F/I) and after treatment with genistein (F/I+Geni). A voltage ramp from -130 mV to +70 mV was applied and current was recorded during the two conditions.

5 Figure 8 is a recording illustrating the effect of quercetin on nasal potential difference (PD) measurement in a healthy human volunteer. Amiloride (50 μ M) was added to block sodium transport as indicated. Conditions were rendered chloride free (Cl free) and chloride secretion was stimulated with isoproterenol (iso; 5 μ M). Quercetin (querc; 10 μ M) was added as indicated.

10 Figure 9 is a recording illustrating the effect of apigenin and kaempferol on nasal PD in mice. Chloride secretion was stimulated with isoproterenol (iso; 5 μ M). and amiloride (50 μ M) was added to block sodium transport as indicated. Under chloride-free conditions (Cl free), apigenin (50 μ M, left panel) and kaempferol (kaemp, 50 μ M, right panel) were added as indicated.

15 Figure 10 is a recording illustrating the effect of genistein, with and without 4-phenylbutyrate, on chloride current in JME cells. The recording was performed at 0 mV holding potential with a 17:150 mM chloride gradient from bath to pipette. The bottom trace is from an untreated cell and the top trace is from a cell that had been incubated in 5 mM 4-phenylbutyrate (4-PB) for two days. Forskolin (10 μ M) and genistein (30 μ M) were added as indicated.

20 Figures 11A-11C are a whole cell patch clamp recording (Figure 11A) and graphs (Figures 11B and 11C) illustrating the effect of forskolin and genistein on HeLa cells infected with a G551D-CFTR-containing adenovirus. Cells were stimulated with forskolin (10 μ M) and genistein (30 μ M), as indicated. The fit of the data with the Goldman equation is shown by the line in Figure 11B. A current variance to mean current plot is shown in Figure 11C.

25 Figure 12A and 12B illustrate the use of representative flavenoids for the treatment of CF patients. Figure 12A shows a recording from a patient with the genotype G551D/ Δ F508. Amiloride, chloride free solution and isoproterenol were added as indicated. The addition of genistein, as indicated, hyperpolarized nasal PD.

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Figure 12B is a graph illustrating the average responses of nasal PD to genistein and quercetin of four CF patients with the G551D mutation. The filled bars show, for comparison, the respective responses in healthy subjects.

Figures 13A-13C illustrate the effect of additional representative
5 flavenoids and isoflavenoids on chloride current in epithelial cells. Figure 13A is a graph showing the stimulation of transepithelial chloride currents by resveratrol (100 μ M), flavanone (100 μ M), flavone (200 μ M), apigenin (20 μ M), apigenin 7-O-neohesperidoside (30 μ M), kaempferol (20 μ M), fisetin (100 μ M), quercetin (30 μ M), rutin (30 μ M), genistein (30 μ M), daidzein (50 μ M), biochanin A (100 μ M) and
10 prunetin (100 μ M) in Calu-3 monolayers. Experiments were performed in the presence of 10 μ M forskolin. Stimulated currents are plotted relative to forskolin stimulated increase (forskolin stimulated currents are 100%). Figure 13B is a recording showing the effect of 7,4'-Dihydroxyflavone on chloride current in unstimulated tissue. This recording shows a dose-dependent stimulation of transepithelial short-circuit current
15 (Isc) across Calu-3 monolayers by 7,4'-Dihydroxyflavone. Increasing concentrations of 7,4'-Dihydroxyflavone (as indicated in μ M) were added to mucosal side and dose-dependently stimulated chloride currents. Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained at 2 mV. Figure 13C is a recording illustrating the effect of trimethoxy-apigenin. This recording shows dose-
20 dependent stimulation of transepithelial short-circuit current (Isc) across Calu-3 monolayers by trimethoxy-apigenin. Increasing concentrations of trimethoxy-apigenin (as indicated in μ M) were added to mucosal side and dose-dependently stimulated chloride currents. Experiment was performed on unstimulated tissue. Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained
25 at 2 mV.

Figure 14 is a recording illustrating the dose-dependent stimulation of transepithelial short-circuit current (Isc) across Calu-3 monolayers by resveratrol. Increasing concentrations of resveratrol (as indicated in μ M) were added to the mucosal perfusion and dose-dependently increased chloride currents. For comparison, currents
30 were further stimulated by serosal addition of 20 μ M forskolin. Stimulated chloride

current was completely blocked by addition of the chloride channel blocker DPC (5 mM). Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained at 2 mV.

Figure 15 is a recording showing L-ascorbic acid and genistein stimulation of transepithelial short-circuit current (Isc) across Calu-3 monolayers. Ascorbic acid (100 μ M) was added as indicated. For comparison, ascorbic acid-stimulated chloride current was subsequently stimulated by the cAMP elevating agonist forskolin (20 μ M, serosal). The CFTR activator genistein (20 mM) was then added to the mucosal perfusion as indicated. Stimulated current was completely blocked by addition of the chloride channel blocker DPC (5 mM), added as indicated. Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained at 2 mV.

Figure 16 is a recording showing L-Ascorbic acid and kaempferol stimulation of transepithelial short-circuit current (Isc) across Calu-3 monolayers. 100 μ M ascorbic acid and forskolin (fsk, 20 μ M, serosal) were added as indicated. The CFTR activator kaempferol (20 μ M) was subsequently added, as indicated. Stimulated current was completely blocked by addition of the chloride channel blocker DPC (5 mM). Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained at 2 mV.

Figure 17 is a recording illustrating the effect of L-ascorbic acid on nasal potential difference in human subjects. Amiloride, chloride-free solution and L-ascorbic acid (100 μ M) were added to the luminal perfusate in the nose, as indicated. The β -adrenergic agonist isoproterenol was also added as indicated. Stimulation was reversed by washing out drugs with NaCl Ringer solution.

Figure 18 is a recording illustrating the stimulation of transepithelial short-circuit current (Isc) across Calu-3 monolayers by addition of 10, 100 and 300 μ M dehydroascorbic acid. Currents were recorded with a serosal-to-mucosal chloride gradient at 0 mV and pulses were obtained at 2mV.

Figure 19 is a recording illustrating the stimulatory effect of 20 μ M genistein on transepithelial short-circuit current (Isc) across 31EG4 mammary epithelial

monolayers. Na currents were blocked by mucosal addition of amiloride (10 mM), and chloride currents were further stimulated by forskolin (20 μ M, serosal), as indicated. Currents were recorded in symmetrical NaCl Ringers solution at 0 mV and pulses were obtained at 2 mV.

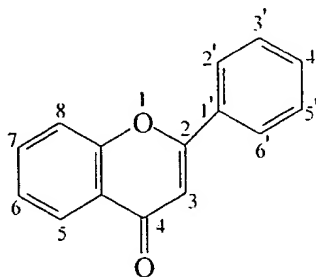
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DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the treatment of diseases characterized by defective chloride transport in epithelial tissues, including cystic fibrosis, and diseases with excessive accumulation of mucus, including cystic fibrosis, chronic bronchitis and asthma. It has been found, within the context of the present invention, that certain flavones and isoflavones, as well as other polyphenolic compounds, are capable of stimulating CFTR-mediated chloride transport in epithelial tissues (*e.g.*, tissues of the airways, intestine, pancreas and other exocrine glands) in a cyclic-AMP independent manner. Ascorbic acid and derivatives thereof may also, or alternatively, be used within such methods. It has further been found, within the context of the present invention, that such compounds stimulate chloride transport in cells with a mutated CFTR (*e.g.*, Δ F508-CFTR or G551D-CFTR). Such therapeutic compounds may be administered to patients afflicted with cystic fibrosis as described herein.

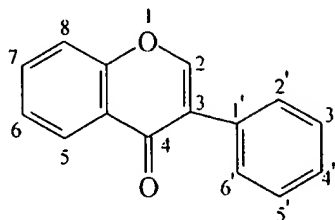
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The term "flavones," as used herein refers to a compound based on the core structure of flavone:



Flavone

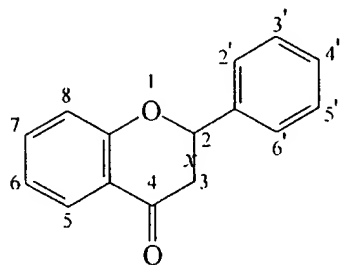
An "isoflavone" is an isomer of a flavone (*i.e.*, the phenyl moiety at position 2 is moved to position 3), and having the core structure shown below:



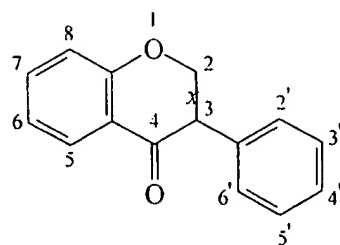
Isoflavone

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Certain flavones and isoflavones have the structure:



or



10 wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond. Stereoisomers and glycoside derivatives of such polyphenolic compounds may also be used within the methods provided herein.

15 Many flavones are naturally-occurring compounds, but synthetic flavones and isoflavones are also encompassed by the present invention. A flavone or isoflavone may be modified to comprise any of a variety of functional groups, such as

hydroxyl and/or ether groups. Preferred flavones comprise one or more hydroxyl groups, such as the trihydroxyflavone apigenin, the tetrahydroxyflavone kaempferol and the pentahydroxyflavone quercetin. Preferred isoflavones comprise one or more hydroxyl and/or methoxy groups, such as the methoxy, dihydroxy isoflavone biochanin

5 A. Genistein is yet another preferred isoflavone for use within the methods provided herein.

Flavones and isoflavones for use within the context of the present invention have the ability to stimulate chloride transport in epithelial tissues. Such transport may result in secretion or absorption of chloride ions. The ability to stimulate

10 chloride transport may be assessed using any of a variety of systems. For example, *in vitro* assays using a mammalian trachea or a cell line, such as the permanent airway cell line Calu-3 (ATCC Accession Number HTB55) may be employed. Alternatively, the ability to stimulate chloride transport may be evaluated within an *in vivo* assay employing a mammalian nasal epithelium. In general, the ability to stimulate chloride

15 transport may be assessed by evaluating CFTR-mediated currents across a membrane by employing standard Ussing chamber (*see* Ussing and Zehrahn, *Acta. Physiol. Scand.* 23:110-127, 1951) or nasal potential difference measurements (*see* Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995). Within such assays, a flavone or isoflavone that stimulates a statistically significant increase in chloride transport at a concentration of

20 about 1 - 300 μ M is said to stimulate chloride transport.

Within one *in vitro* assay, the level of chloride transport may be evaluated using mammalian pulmonary cell lines, such as Calu-3 cells, or primary bovine tracheal cultures. In general, such assays employ cell monolayers, which may be prepared by standard cell culture techniques. Within such systems, CFTR-mediated

25 chloride current may be monitored in an Ussing chamber using intact epithelia. Alternatively, chloride transport may be evaluated using epithelial tissue in which the basolateral membrane is permeabilized with *Staphylococcus aureus* α -toxin, and in which a chloride gradient is imposed across the apical membrane (*see* Illek et al., *Am. J. Physiol.* 270:C265-75, 1996). In either system, chloride transport is evaluated in the

30 presence and absence of a test compound (*i.e.*, a flavone or isoflavone), and those

compounds that stimulate chloride transport as described above may be used within the methods provided herein.

Within another *in vitro* assay for evaluating chloride transport, cells are transfected with a chloride channel gene (*e.g.*, CFTR) having a mutation associated with cystic fibrosis. Any CFTR gene that is altered relative to the normal human sequence provided in SEQ ID NO:1, such that the encoded protein contains a mutation associated with cystic fibrosis, may be employed within such an assay. The most common disease-causing mutation in cystic fibrosis is a deletion of phenylalanine at position 508 in the CFTR protein (Δ F508-CFTR; SEQ ID NO:4). Accordingly, the use of a CFTR gene encoding Δ F508-CFTR is preferred. However, genes encoding other altered CFTR proteins (*e.g.*, G551D-CFTR; containing a glycine to aspartate point mutation at position 551; SEQ ID NO:6) may also be used. Cells such as NIH 3T3 fibroblasts may be transfected with an altered CFTR gene, such as Δ F508-CFTR, using well known techniques (*see* Anderson et al., *Science* 25:679-682, 1991). The effect of a compound on chloride transport in such cells may be evaluated by monitoring CFTR-mediated currents using the patch clamp method (*see* Hamill et al., *Pflugers Arch.* 391:85-100, 1981) with and without compound application.

Within another *in vitro* assay, a mutant CFTR may be microinjected into cells such as *Xenopus* oocytes. Chloride conductance mediated by the CFTR mutant in the presence and absence of a test compound may be monitored with the two electrode voltage clamp method (*see* Miledi et al., *Proc. R. Soc. Lond. Biol.* 218:481-484, 1983).

Alternatively, such assays may be performed using a mammalian trachea, such as a primary cow tracheal epithelium using the Ussing chamber technique as described above. Such assays are performed in the presence and absence of test compound to identify flavone and isoflavones that stimulate chloride transport.

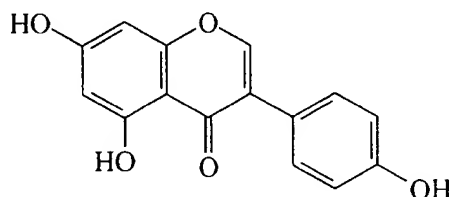
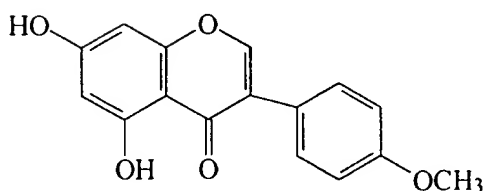
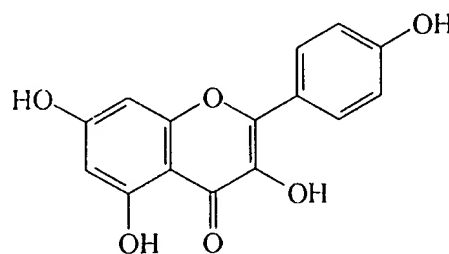
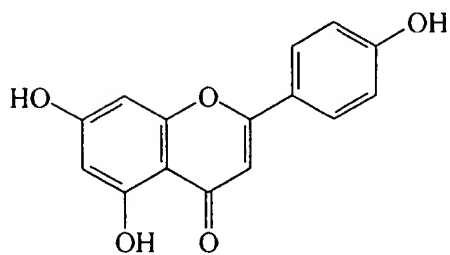
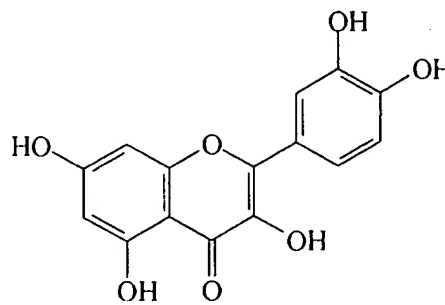
Any of the above assays may be performed following pretreatment of the cells with a substance that increases the concentration of CFTR mutants in the plasma membrane. Such substances include chemical chaperones, which support correct trafficking of the mutant CFTR to the membrane, and compounds that increase expression of CFTR in the cell (*e.g.*, transcriptional activators). A "chemical

chaperone," as used herein is any molecule that increases trafficking of proteins to a cell membrane. More specifically, a chemical chaperone within the context of the present invention increases trafficking of a mutant CFTR (e.g., the $\Delta 508$ -CFTR and/or G551D-CFTR) to the membrane by a statistically significant amount. Chemical chaperones for use herein include, but are not limited to, glycerol, dimethylsulfoxide, trimethylamine N-oxide, taurine, methylamine and deoxyspergualin (see Brown et al., *Cell Stress Chaperones* 1:117-125, 1996; Jiang et al., *Amer. J. Physiol.-Cell Physiol.* 44:C171-C178, 1998). Compounds that increase expression of CFTR in the cell include 4-phenylbutyrate (Rubenstein et al., *J. Clin. Invest.* 100:2457-2465, 1997) and sodium butyrate (Cheng et al., *Am. J. Physiol.* 268:L615-624, 1995). Other compounds that increase the level of CFTR in the plasma membrane (by increasing correct trafficking and/or expression of the CFTR) may be readily identified using well known techniques, such as immunohistochemical techniques, to evaluate effects on levels of plasma membrane CFTR.

In vivo, chloride secretion may be assessed using measurements of nasal potential differences in a mammal, such as a human or a mouse. Such measurements may be performed on the inferior surface of the inferior turbinate following treatment of the mucosal surface with a test compound during perfusion with the sodium transport blocker amiloride in chloride-free solution. The nasal potential difference is measured as the electrical potential measured on the nasal mucosa with respect to a skin electrode placed on a slightly scratched skin part (see Alton et al., *Eur. Respir. J.* 3:922-926, 1990) or with respect to a subcutaneous needle (see Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995). Nasal potential difference is evaluated in the presence and absence of test compound, and those compounds that results in a statistically significant increase in nasal potential difference stimulate chloride transport.

Compounds as provided herein may generally be used to chloride transport within any of a variety of CFTR-expressing epithelial cells. CFTR is expressed in many epithelial cells, including intestinal, airway, pancreas, gallbladder, sweat duct, salivary gland and mammary epithelia. All such CFTR-expressing organs are subject to stimulation by the compounds provided herein.

As noted above, any flavone or isoflavone that stimulates chloride transport within at least one of the above assays may be used for therapy of cystic fibrosis, other diseases characterized by abnormally high mucus accumulation in the airways or intestinal disorders such as constipation. Preferred therapeutic compounds include flavones and isoflavones that occur naturally in plants and are part of the human diet. Preferred compounds include genistein (4',5,7-trihydroxyisoflavone), as well as quercetin (3,3',4',5,7-pentahydroxyflavone), apigenin (4',5,7-trihydroxyflavone), kaempferol (3,4',5,7-tetrahydroxyflavone) and biochanin A (4'-methoxy-5,7-dihydroxyisoflavone), as depicted below:

**Genistein****Biochanin A****Kaempferol****Apigenin****Quercetin**

Other suitable therapeutic compounds may be identified using the representative assays as described herein. Additional representative flavones and isoflavones include flavanone, flavone, dihydroxyflavone, trimethoxy-apigenin, apigenin 7-O-neohesperidoside, fisetin, rutin, daidzein and prunetin. Representative flavones and isoflavones are summarized in Tables I and II.

Table I
Flavonoids

No.	Name	X	C3	C5	C7	C3'	C4'
1	Apigenin	=		OH	OH		OH
2	Apigenin 7-O-neohesperidoside	=		OH	ONeo		OH
3	Dihydroxyflavone	=		OH			OH
4	Flavone	=					
5	Flvanone	-					
6	Fisetin	=	OH		OH	OH	OH
7	Kaempferol	=	OH	OH	OH		OH
8	Quercetin	=	OH	OH	OH	OH	OH
9	Rutin	=	ORut		OH	OH	OH
10	Trimethoxy-apigenin	=	H	OCH3	OCH3		OCH3

10 where = a double bond, - is a single bond, ONeo is Neohesperidoside, ORut is rutinoside, OCH3 is methoxy, OH is hydroxy

Table II
Isoflavonoids

No.	Name	X	C5	C7	C4'
11	Biochanin	=	OH	OH	OCH3
12	Daidzein	=		OH	OH
13	Genistein	=	OH	OH	OH
14	Prunetin	=	OH	OCH3	OH

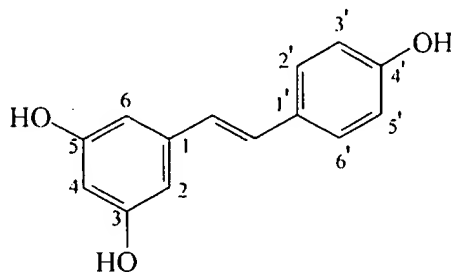
15 where = a double bond, - is a single bond, ONeo is Neohesperidoside, ORut is rutinoside, OCH3 is methoxy, OH is hydroxy.

Genistein, quercetin, apigenin, kaempferol, biochanin A and other flavones and isoflavones may generally be prepared using well known techniques, such

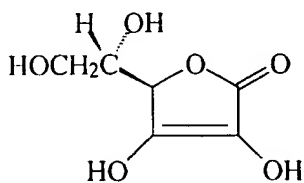
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as those described by Shakhova et al., *Zh. Obshch. Khim.* 32:390, 1962; Farooq et al., *Arch. Pharm.* 292:792, 1959; and Ichikawa et al., *Org. Prep. Prog. Int.* 14:183, 1981. Alternatively, such compounds may be commercially available (e.g., from Indofine Chemical Co., Inc., Somerville, NJ or Sigma-Aldrich, St. Louis, MO). Further
5 modifications to such compounds may be made using conventional organic chemistry techniques, which are well known to those of ordinary skill in the art.

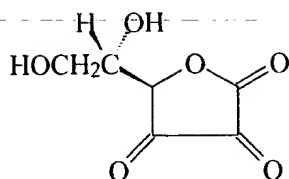
As noted above, other polyphenolic compounds may be used within the methods provided herein. For example, trihydroxystilbenes such as resveratrol (trans-3,5,4'-trihydroxystilbene) may be employed. Resveratrol is a polyphenolic compound
10 having the following structure:



Other compounds that may be used within the methods provided herein are ascorbic acid and derivatives thereof. Such compounds include L-ascorbic acid (L-xyloascorbic acid), dehydroascorbic acid (L-threo-2,3-Hexodiulosonic acid γ -lactone)
15 and salts of the foregoing acids.



L-Ascorbic Acid



Dehydroascorbic Acid

Within certain preferred embodiments, ascorbic acid or a derivative thereof is used in combination with a polyphenolic compound as described above. Certain representative combinations include ascorbic acid and one or more flavenoids and/or isoflavenoids (such as genistein and ascorbic acid; and kaempferol and ascorbic acid). Ascorbic acid may generally be used to treat or prevent genetic loss of chloride secretory function (*e.g.*, cystic fibrosis), as well as other related loss or reduced chloride secretory function (*e.g.*, intestinal constipation, dry eye syndrome and obstructive airway diseases).

For *in vivo* use, a therapeutic compound as described herein is generally incorporated into a pharmaceutical composition prior to administration. Within such compositions, one or more therapeutic compounds as described herein are present as active ingredient(s) (*i.e.*, are present at levels sufficient to provide a statistically significant effect on nasal potential difference, as measured using a representative assay as provided herein). A pharmaceutical composition comprises one or more such compounds in combination with any physiologically acceptable carrier(s) and/or excipient(s) known to those skilled in the art to be suitable for the particular mode of administration. In addition, other pharmaceutically active ingredients (including other therapeutic agents) may, but need not, be present within the composition.

Within certain methods provided herein, a flavone or isoflavone may be combined with a substance that increases the concentration of CFTR mutants in the plasma membrane of a cell. As noted above, such substances include chemical chaperones, which support correct trafficking of the mutant CFTR to the membrane, and compounds that increase expression of CFTR in the membrane. These substances may be contained within the same pharmaceutical composition or may be administered separately. Preferred chemical chaperones include glycerol, dimethylsulfoxide,

trimethylamine N-oxide, taurin, methylamine and deoxyspergualin, and compounds that increase expression of CFTR in the membrane include 4-phenylbutyrate and sodium butyrate. The use of flavenoid and/or isoflavenoid compounds, as described herein, in combination with such substances may increase mutant CFTR activity, and
5 ameliorate symptoms of cystic fibrosis.

Administration may be achieved by a variety of different routes. One preferred route is oral administration of a composition such as a pill, capsule or suspension. Such compositions may be prepared according to any method known in the art, and may comprise any of a variety of inactive ingredients. Suitable excipients for
10 use within such compositions include inert diluents (which may be solid materials, aqueous solutions and/or oils) such as calcium or sodium carbonate, lactose, calcium or sodium phosphate, water, arachis oil, peanut oil liquid paraffin or olive oil; granulating and disintegrating agents such as maize starch, gelatin or acacia and/or lubricating agents such as magnesium stearate, stearic acid or talc. Other inactive ingredients that
15 may, but need not, be present include one or more suspending agents (*e.g.*, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia), thickeners (*e.g.*, beeswax, paraffin or cetyl alcohol), dispersing or wetting agents, preservatives (*e.g.*, antioxidants such as ascorbic acid), coloring agents, sweetening agents and/or flavoring
20 agents.

A pharmaceutical composition may be prepared with carriers that protect active ingredients against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems, and biodegradable,
25 biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others known to those of ordinary skill in the art.

Particularly preferred are methods in which the therapeutic compound(s) are directly administered as a pressurized aerosol or nebulized formulation to the
30 patient's lungs via inhalation. Such formulations may contain any of a variety of known

aerosol propellants useful for endopulmonary and/or intranasal inhalation administration. In addition, water may be present, with or without any of a variety of cosolvents, surfactants, stabilizers (e.g., antioxidants, chelating agents, inert gases and buffers). For compositions to be administered from multiple dose containers, antimicrobial agents are typically added. Such compositions are also generally filtered and sterilized, and may be lyophilized to provide enhanced stability and to improve solubility.

Pharmaceutical compositions are administered in an amount, and with a frequency, that is effective to inhibit or alleviate the symptoms of cystic fibrosis and/or to delay the progression of the disease. The effect of a treatment may be clinically determined by nasal potential difference measurements as described herein. The precise dosage and duration of treatment may be determined empirically using known testing protocols or by testing the compositions in model systems known in the art and extrapolating therefrom. Dosages may also vary with the severity of the disease. A pharmaceutical composition is generally formulated and administered to exert a therapeutically useful effect while minimizing undesirable side effects. In general, an oral dose ranges from about 200 mg to about 1000 mg, which may be administered 1 to 3 times per day. Compositions administered as an aerosol are generally designed to provide a final concentration of about 10 to 50 μ M at the airway surface, and may be administered 1 to 3 times per day. It will be apparent that, for any particular subject, specific dosage regimens may be adjusted over time according to the individual need.

As noted above, a pharmaceutical composition may be administered to a mammal to stimulate chloride transport, and to treat cystic fibrosis. Patients that may benefit from administration of a therapeutic compound as described herein are those afflicted with cystic fibrosis. Such patients may be identified based on standard criteria that are well known in the art, including the presence of abnormally high salt concentrations in the sweat test, the presence of high nasal potentials, or the presence of a cystic fibrosis-associated mutation. Activation of chloride transport may also be beneficial in other diseases that show abnormally high mucus accumulation in the airways, such as asthma and chronic bronchitis. Similarly, intestinal constipation may

benefit from activation of chloride transport by a flavone or isoflavone as provided herein.

Summary of Sequence Listing

5

SEQ ID NO:1 is a DNA sequence encoding human CFTR.

SEQ ID NO:2 is an amino acid sequence of human CFTR.

SEQ ID NO:3 is a DNA sequence encoding human CFTR with the $\Delta F508$ mutation.

10

SEQ ID NO:4 is an amino acid sequence of human CFTR with the $\Delta F508$ mutation.

SEQ ID NO:5 is a DNA sequence encoding human CFTR with the G551D mutation.

15 G551D mutation.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

5 Stimulation of Chloride Transport by Representative
 Flavones and Isoflavones in Airway Cells

 This Example illustrates the use of the representative compounds apigenin, quercetin and biochanin A to enhance chloride secretion in Calu-3 human pulmonary cultures or in primary bovine tracheal cultures.

10 A Calu-3 cell monolayer was prepared in an Ussing chamber as described by Illek et al., *Am. J. Physiol.* 270:C265-275, 1996. The basolateral membrane was permeabilized with α -toxin and a chloride gradient was applied across the apical membrane as a driving force (*see* Illek et al., *Am. J. Physiol.* 270:C265-C275, 1996). The tissue was first stimulated with cAMP (100 μ M), and then with a
15 representative flavone or isoflavone.

 As shown in Figures 1 and 2, subsequent addition of apigenin or quercetin further stimulated chloride current. Figure 1 illustrates the short circuit current across the Calu-3 cell monolayer before and after addition of apigenin (50 μ M). Figure 2 illustrates the effect of quercetin (30 μ M) on chloride current across a Calu-3
20 monolayer. In both cases, the flavone stimulated chloride current beyond the stimulation achieved by cAMP.

 Figure 3 illustrates the results of a related experiment to evaluate the dose-dependent stimulation of transepithelial chloride secretion by quercetin across a primary bovine tracheal epithelium. The epithelial cells were first treated with
25 amiloride (50 μ M), and then with quercetin at the indicated concentrations. The dose-response relation yielded a half maximal stimulation at 12.5 μ M. At high concentrations of quercetin, the current was blocked. Current was fully inhibited by the CFTR channel blocker diphenylcarboxylate (DPC, 5 mM).

 To evaluate the effect of biochanin A, a Calu-3 cell monolayer was
30 prepared and permeabilized as described above. The tissue was first stimulated with

forskolin (Fsk, 10 μ M). The effect of biochanin A (Bio, 100 and 300 μ M) on short-circuit current (I_{sc}) across the Calu-3 monolayer was evaluated in an Ussing chamber. As shown in Figure 4, biochanin A further stimulated chloride secretion.

5

Example 2

Activation of Mutant CFTR by Representative Flavones and Isoflavones

This Example illustrates the use of the representative compounds apigenin, quercetin and genistein to activate Δ F508-CFTR and G551D-CFTR in
10 different cell types.

A cell-attached single channel patch clamp recording was obtained from a 3T3 cell expressing Δ F508-CFTR as described by Hamill et al., *Pflugers Arch.* 391:85-100, 1981 and Fischer and Machen, *J. Gen. Physiol.* 104:541-566, 1994. As shown in Figure 5, stimulation of the cell with 10 μ M forskolin did not activate Δ F508-CFTR channel, but addition of genistein (50 μ M) or apigenin (50 μ M, where indicated
15 by boxes) induced Δ F508-CFTR channel openings, and removal of these compounds inactivated the channels. The holding potential was 75 mV, and channel openings were upward.

Figure 6 presents a whole cell patch clamp recording on an airway epithelial cell homozygous for Δ F508-CFTR (cell type: JME cell, see Jeffersen et al., *Am. J. Physiol.* 259:L496-L505, 1990). Before the measurement, the cell was incubated for 2 days in 5 mM 4-phenylbutyrate to enhance Δ F508-CFTR expression in the plasma membrane (Rubenstein & Zeitlin, *Ped. Pulm. Suppl.* 12:234, 1995). Measurements were performed as described by Fischer et al., *J. Physiol. Lond.* 489:745-754, 1995. Addition of 30 μ M quercetin activated chloride current in the
20 whole cell mode, which was further stimulated by forskolin. The holding potential was -60 mV.

The effect of genistein on chloride current in a *Xenopus* oocyte expressing G551D-CFTR was measured with the two-electrode voltage clamp
30 technique (see Miledi et al., *Proc. R. Soc. Lond. Biol.* 218:481-484, 1983). G551D-

CFTR (2 ng in 50-nL of water) was injected into the oocyte. Current was first stimulated with forskolin (10 μ M) and isobutylmethylxanthine (IBMX; 2 mM). Genistein (50 μ M) was found to further activate chloride currents. As shown in Figure 7, genistein increased conductance and shifted reversal potential to the right, which is indicative of a stimulated chloride current.

Example 3

Effect of Representative Flavones on Nasal Potential Difference

This Example illustrates the *in vivo* use of quercetin, apigenin and kaempferol to activate the nasal potential difference in humans and mice.

The effect of quercetin on nasal potential difference (PD) measurement in a healthy human volunteer was measured as described by Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995. Under conditions where sodium transport was blocked with amiloride (50 μ M) and chloride secretion was stimulated under chloride-free conditions with isoproterenol (5 μ M), quercetin (10 μ M) stimulated nasal PD further (Figure 8).

The effect of apigenin and kaempferol on nasal PD in mice was evaluated using a method similar to that employed for measurements in humans, except that a plastic tube of approximately 0.1 mm diameter was used as an exploring nasal electrode. The plastic tube was perfused with test solutions at approximately 10 μ L/min. After blocking sodium transport with amiloride (50 μ M) and during stimulation of chloride secretion with isoproterenol (iso:5 μ M) under chloride-free conditions, apigenin (50 μ M, left panel) and kaempferol (kaemp. 50 μ M, right panel) further stimulated nasal PD.

These results show that the representative flavenoids quercetin, apigenin, kaempferol and biochanin A stimulate chloride transport across epithelial tissues derived from the airways *in vitro*, and across nasal epithelium *in vivo*. The results also show that the CFTR mutants Δ F508 and G551D can be activated by the representative compounds genistein and apigenin.

Example 4

Effect of Genistein on Chloride Current in Cells Expressing a Mutant CFTR

This Example illustrates the ability of the representative isoflavone
5 genistein to activate chloride current in cells expressing a mutant CFTR.

In one experiment, genistein was used in combination with 4-phenylbutyrate. Chloride current was measured in JME cells (human nasal epithelial cell line homozygous for the $\Delta 508$ mutation of CFTR; see Jefferson et al., *Am. J. Physiol.* 259:L496-505, 1990). The recording was performed at 0 mV holding potential
10 with a 17:150 mM chloride gradient from bath to pipette. Under these conditions, the recorded current, shown in Figure 10, is chloride current (Illek and Fischer, *Am. J. Physiol. (Lung Cell. Mol. Physiol.)*:L902-910, 1998). The bottom trace in Figure 10 is from an untreated cell. Neither forskolin (10 μ M nor genistein (30 μ M activated current. The top tracing in Figure 10 is from a cell that had been incubated in 5 mM 4-phenylbutyrate (4-PB) for two days (Rubenstein et al., *J. Clin. Invest.* 100:2457-2465,
15 1997). After 4-PB treatment, chloride current was stimulated by forskolin (by on average 30.3 ± 19.4 pS/pF, n=6), and further activated by genistein (to an average 105 ± 84 pS/pF) in a CF cell with the $\Delta 508$ -CFTR mutation. These results further demonstrate the ability of a flavenoid compound to optimize chloride currents elicited
20 in CF cells by other means.

Within another experiment, HeLa cells infected with the G551D-CFTR-containing adenovirus were investigated in the patch clamp mode. Stimulation of the cell with forskolin (10 μ M) stimulated only a very small current (Figures 11A and 11B). On average, forskolin-stimulated conductance was 9.5 ± 5 pS/pF (n=4).
25 Additional stimulation with genistein (30 μ M) stimulated significant chloride currents, which were time- and voltage-independent (Figure 11B) and well fitted with the Goldman equation (line in Figure 11B; Illek and Fischer, *Am. J. Physiol. (Lung Cell. Mol. Physiol.)*:L902-910, 1998), which are characteristics of CFTR-mediated currents. Average forskolin + genistein-activated conductance was 120 ± 30 pS/pF (n=4).
30 Current variance to mean current plot (Figure 11C) were used to calculate the average

open probability (P_o shown on top of axis) of the population of channels carrying the total current (as described in Illek and Fischer, *Am. J. Physiol. (Lung Cell. Mol. Physiol.)*:L902-910, 1998). During forskolin stimulation, maximal P_o reached was 0.04 (open circles) and after additional stimulation with genistein P_o reached a maximum of 0.42 in this recording. On average, after forskolin stimulation, $P_o = 0.05 \pm 0.02$ and after forskolin + genistein stimulation $P_o = 0.54 \pm 0.12$. For comparison, wild type CFTR expressed in HeLa cells and recorded under the same conditions resulted in $P_o = 0.36 \pm 0.05$ (n=3) after forskolin stimulation and $P_o = 0.63 \pm 0.16$ after forskolin + genistein treatment.

10

Example 5

Effect of Representative Flavones on Nasal Potential Difference in CF Patients

This Example illustrates the *in vivo* use of quercetin and genistein to activate the nasal potential difference in CF patients bearing the G551D mutation.

Measurements were performed on patients as described by Alton et al., *Eur. Respir. J.* 3:922-926, 1990; Illek and Fischer, *Am. J. Physiol. (Lung Cell. Mol. Physiol.)*:L902-910, 1998; and Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995). The results are presented in Figures 12A and 12B. Figure 12A shows a recording from a patient with the genotype G551D/ Δ F508. Initial treatment with amiloride and chloride free solution had the purpose to isolate and amplify the chloride selective potentials. Addition of the beta-adrenergic agonist isoproterenol showed no effect, which is typical for CF patients (Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995). However, addition of genistein hyperpolarized nasal PD. Average responses of nasal PD to genistein and quercetin of four CF patients with the G551D mutation are shown in Figure 12B (open bars). The filled bars show for comparison the respective responses in healthy subjects. The genotypes of the 4 CF patients were: two G551D/ Δ F508, one G551D/G551D and one G551D/unknown. Responses are most likely due to the G551D mutation because the homozygous G551D patient responded

not different compared to the heterozygous G551D patients. Genistein and quercetin responses of nasal PD in CF patients were significant ($p < 0.05$).

These results demonstrate that CFTR mutations are sensitive to flavenoid treatment, and provide additional evidence for therapeutic benefit of such compounds for the treatment of cystic fibrosis.

Example 6

Effect of Additional Representative Polyphenolic Compounds on

10

Epithelial Cell Chloride Currents

This Example illustrates the effect of further flavenoids and isoflavenoids on chloride currents in airway epithelial cells.

Airway epithelial cells were prestimulated with 10 μM forskolin. The percent increase in chloride current was then determined following treatment with a series of polyphenolic compounds. Figure 13A summarizes the stimulatory effect of these compounds. On average, chloride currents were further stimulated by resveratrol (100 μM) to 135%, by flavanone (100 μM) to 140%, by flavone (200 μM) to 128%, by apigenin (20 μM) to 241%, by apigenin 7-O-neohesperidoside (30 μM) to 155%, by kaempferol (20 μM) to 182%, by fisetin (100 μM) to 108%, by quercetin (30 μM) to 169%, by rutin (30 μM) to 149%, by genistein (30 μM) to 229%, by daidzein (50 μM) to 162%, by biochanin A (100 μM) to 139% and by prunetin (100 μM) to 161%.

The stimulatory effect of 7,4'-Dihydroxyflavone is shown in Figure 13B. Addition of 7,4'-Dihydroxyflavone to the mucosal perfusion dose-dependently stimulated transepithelial Cl^- currents in unstimulated Calu-3 monolayers. This experiment was performed using unstimulated tissue.

The stimulatory effect of trimethoxy-apigenin is shown in Figure 13C. Addition of trimethoxy-apigenin to the mucosal perfusion dose-dependently stimulated transepithelial Cl^- currents in unstimulated Calu-3 monolayers. Kinetic analysis is depicted on the right panel and estimated half maximal stimulatory dose was 11.7 μM .

These results indicate that a variety of polyphenolic compounds stimulate chloride currents in epithelial cells.

5

Example 7

Effect of Resveratrol on Chloride Currents

This Example illustrates the stimulatory effect of resveratrol on transepithelial chloride currents.

Unstimulated Calu-3 monolayers were treated with increasing concentrations of resveratrol. Figure 14 shows the recording generated following the addition of resveratrol to the mucosal perfusion dose-dependently stimulated transepithelial chloride currents in unstimulated Calu-3 monolayers. For comparison, currents were further stimulated by serosal addition of forskolin. The stimulated chloride current was completely blocked by the Cl channel blocker DPC. These results indicate that resveratrol stimulates transepithelial chloride transport.

15

Example 8

Effect of Ascorbic Acid and Dehydroascorbic Acid on Chloride Currents

This Example illustrates the stimulatory effect of ascorbic acid and dehydroascorbic acid on transepithelial chloride current.

Unstimulated Calu-3 monolayers were stimulated with L-ascorbic acid, as shown in Figure 15. Addition of L-ascorbic acid to the mucosal or serosal perfusion very effectively stimulated transepithelial chloride secretion in unstimulated Calu-3 monolayers. For comparison, chloride currents were further stimulated by serosal addition of forskolin. In the continued presence of L-ascorbic acid and forskolin, it is remarkable that addition of genistein further stimulated chloride currents. These results indicate that genistein serves as a potent drug that is able to hyperstimulate chloride secretion and thereby maximize chloride transport across epithelia. The stimulated chloride current was completely blocked by the chloride channel blocker DPC.

20
25
30

The stimulatory effect of L-ascorbic acid is also shown in Figure 16. Addition of 100 μ M L-ascorbic acid to the mucosal or serosal perfusion very effectively stimulated transepithelial chloride currents in unstimulated Calu-3 monolayers. For comparison, ascorbic acid-stimulated chloride currents were stimulated by the cAMP elevating agonist forskolin (20 μ M, serosal). Under these stimulated conditions kaempferol further hyperstimulated chloride currents. The stimulated chloride current was completely blocked by the chloride channel blocker DPC (5 mM).

The stimulatory effect of dehydroascorbic acid is shown in Figure 18. Addition of dehydroascorbic acid at 10, 100 or 300 μ M to the mucosal and serosal perfusion effectively stimulated transepithelial chloride currents in unstimulated Calu-3 monolayers. Stimulated Cl currents returned to baseline after 5-15 min.

15 Example 9

Effect of Ascorbic Acid on Chloride Currents *in vivo*

This Example illustrates the stimulatory effect of ascorbic acid on human nasal potential difference.

Nasal potential difference measurement was performed on a human volunteer according to a protocol by Knowles et al., *Hum. Gene Therapy* 6:445-455, 1995. Addition of L-ascorbic acid (100 μ M) to the luminal perfusate in the nose (in the presence of amiloride (blocks Na currents) and in chloride-free solution) hyperpolarized nasal potential difference (PD) by 6.3 mV (Figure 17). Addition of the β -adrenergic agonist isoproterenol further hyperpolarized nasal PD. Stimulation was reversed by washing out drugs with NaCl Ringer solution. These results demonstrate the ability of ascorbic acid to stimulate chloride transport in epithelia in humans.

Example 10Effect of Genistein on Chloride Currents in Mammary Epithelia

This Example illustrates the stimulatory effect of genistein in mammary epithelial cells.

5 The stimulation of transepithelial short-circuit current (I_{sc}) across 31EG4 mammary epithelial monolayers by addition of 20 μ M genistein is shown in Figure 19. Na currents were blocked by mucosal addition of amiloride (10 mM). Chloride currents were further stimulated by forskolin (20 μ M, serosal). Currents were recorded in symmetrical NaCl Ringers solution at 0 mV and pulses were obtained at 2
10 mV.

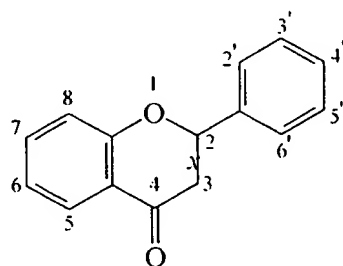
From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the
15 invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

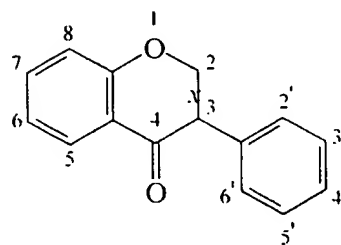
1. A method for enhancing chloride transport in epithelial cells, comprising contacting epithelial cells with a compound selected from the group consisting of flavones and isoflavones, wherein the compound is capable of stimulating chloride transport, and wherein the compound is not genistein.

2. A method according to claim 1, wherein the compound is:

(a) a polyphenolic compound having the general formula:



or



wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond; or

(b) a stereoisomer or glycoside derivative of any of the foregoing polyphenolic compounds.

3. A method according to claim 1, wherein the compound is selected from the group consisting of quercetin, apigenin, kaempferol, biochanin A, flavanone, flavone,

dihydroxyflavone, trimethoxy-apigenin, apigenin, 7-O-neohesperidoside, fisetin, rutin, daidzein and prunetin.

4. A method for enhancing chloride transport in epithelial cells, comprising contacting epithelial cells with a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid.

5. A method according to claim 1 or claim 4, wherein the epithelial cells are airway epithelial cells.

6. A method according to claim 5, wherein the airway epithelial cells are present in a mammal.

7. A method according to claim 6, wherein the compound is administered orally.

8. A method according to claim 6, wherein the compound is administered by inhalation.

9. A method according to claim 1 or claim 4, wherein the epithelial cells are intestinal cells.

10. A method according to claim 9, wherein the intestinal epithelial cells are present in a mammal.

11. A method according to claim 10, wherein the compound is administered orally.

12. A method according to claim 1 or claim 4, wherein the epithelial cells are pancreas, gallbladder, sweat duct, salivary gland or mammary epithelial cells.

13. A method according to claim 12, wherein the intestinal epithelial cells are present in a mammal.

14. A method according to claim 1 or claim 4, wherein the cells are further contacted with a substance that increases (a) trafficking of a CFTR to the plasma membrane of the cells; and/or (b) expression of a CFTR in the cells.

15. A method according to claim 1 or claim 4, wherein the compound is present within a pharmaceutical composition comprising a physiologically acceptable carrier or excipient.

16. A method according to claim 1 or claim 4, wherein the epithelial cells produce a mutated CFTR protein.

17. A method according to claim 16, wherein the mutated CFTR protein has a deletion at position 508 or a point mutation at position 551.

18. A method according to claim 1 or claim 4, wherein the pharmaceutical composition further comprises a substance that increases (a) trafficking of a CFTR to the plasma membrane of the cells; and/or (b) expression of a CFTR in the cells.

19. A method according to claim 18, wherein the substance increases expression of a CFTR in the cells and is 4-phenylbutyrate or sodium butyrate.

20. A method according to claim 18, wherein the substance is a chemical chaperone that increases trafficking of a CFTR to the plasma membrane of the cells, and wherein the compound is selected from the group consisting of glycerol, dimethylsulfoxide, trimethylamine N-oxide, taurin, methylamine and deoxyspergualin.

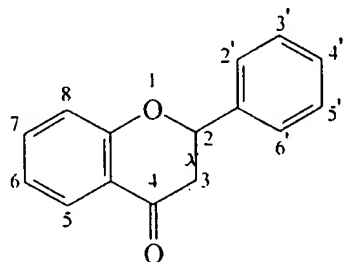
21. A method according to claim 1, wherein the cells are further contacted with a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid.
22. A method according to claim 21, wherein the cells are contacted with a polyphenolic compound and ascorbic acid.
23. A method according to claim 22, wherein the polyphenolic compound is genistein, daidzein or prunetin.
24. A method for enhancing chloride transport in epithelial cells, comprising contacting epithelial cells with genistein, wherein the epithelial cells produce a mutated CFTR protein.
25. A method according to claim 24, wherein the mutated CFTR protein is G551D-CFTR or Δ F508-CFTR.
26. A method according to claim 24, wherein the epithelial cells are further contacted with a substance that increases (a) trafficking of a CFTR to the plasma membrane of the cells; and/or (b) expression of a CFTR in the cells.
27. A method according to claim 26, wherein the substance increases expression of a CFTR in the cells and is 4-phenylbutyrate or sodium butyrate.
28. A method according to claim 26, wherein the substance is a chemical chaperone that increases trafficking of a CFTR to the plasma membrane of the cells, and wherein the compound is selected from the group consisting of glycerol, dimethylsulfoxide, trimethylamine N-oxide, taurin, methylamine and deoxyspergualin.

29. A method for enhancing chloride transport in epithelial cells, comprising contacting epithelial cells with genistein and a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid.

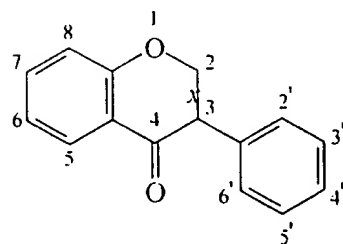
30. A method for treating cystic fibrosis in a mammal, comprising administering to a mammal one or more compounds selected from the group consisting of flavones and isoflavones, wherein the compound is capable of stimulating chloride secretion, and thereby treating cystic fibrosis in the mammal.

31. A method according to claim 30, wherein the compound is:

(a) a polyphenolic compound having the general formula:



or



wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond; or

(b) a stereoisomer or glycoside derivative of any of the foregoing polyphenolic compounds.

32. A method according to claim 31, wherein the compound is selected from the group consisting of quercetin, apigenin, kaempferol, biochanin A, flavanone,

flavone, dihydroxyflavone, trimethoxy-apigenin, apigenin-7-O-neohesperidoside, fisetin, rutin, daidzein and prunetin.

33. A method according to claim 31, wherein the compound is genistein.

34. A method for treating cystic fibrosis in a mammal, comprising administering to a mammal one or more compounds selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid.

35. A method according to claim 30 or claim 34, wherein the compound is administered orally.

36. A method according to claim 30 or claim 34, wherein the compound is administered by inhalation.

37. A method according to claim 30 or claim 34, wherein a substance is further administered to the mammal, such that the substance increases (a) trafficking of a CFTR to the plasma membrane of epithelial cells; and/or (b) expression of a CFTR in epithelial cells.

38. A method according to claim 37, wherein the substance increases expression of a CFTR in the cells and is 4-phenylbutyrate or sodium butyrate.

39. A method according to claim 37, wherein the substance is a chemical chaperone that increases trafficking of a CFTR to the plasma membrane of the cells, and wherein the compound is selected from the group consisting of glycerol, dimethylsulfoxide, trimethylamine N-oxide, taurin, methylamine and deoxyspergualin.

40. A method according to claim 30, wherein the compound is present within a pharmaceutical composition comprising a physiologically acceptable carrier or excipient.

41. A method according to claim 40, wherein the pharmaceutical composition further comprises a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid.

42. A method according to claim 40, wherein the pharmaceutical composition further comprises a substance that increases (a) trafficking of a CFTR to the plasma membrane of epithelial cells; and/or (b) expression of a CFTR in epithelial cells.

43. A method according to claim 40, wherein the substance increases expression of a CFTR in the cells and is 4-phenylbutyrate or sodium butyrate.

44. A method according to claim 42, wherein the substance is a chemical chaperone that increases trafficking of a CFTR to the plasma membrane of the cells, and wherein the compound is selected from the group consisting of glycerol, dimethylsulfoxide, trimethylamine N-oxide, taurin, methylamine and deoxyspergualin.

45. A method for increasing chloride ion conductance in airway epithelial cells of a patient afflicted with cystic fibrosis, wherein the patient's CFTR protein has a deletion at position 508, the method comprising administering to a mammal one or more compounds selected from the group consisting of flavones and isoflavones, wherein the compound is capable of stimulating chloride secretion.

46. A method according to claim 45, wherein the compound is genistein.

47. A method according to claim 45, wherein the compound is quercetin.

48. A method for increasing chloride ion conductance in airway epithelial cells of a patient afflicted with cystic fibrosis, wherein the patient's CFTR protein has a mutation at position 551, the method comprising administering to a mammal one or more compounds selected from the group consisting of flavones and isoflavones, wherein the compound is capable of stimulating chloride secretion.

49. A method according to claim 48, wherein the compound is genistein.

50. A method according to claim 48, wherein the compound is quercetin.

51. A pharmaceutical composition for treatment of cystic fibrosis, comprising:

(a) one or more flavones or isoflavones capable of stimulating chloride secretion;

(b) one or more of:

(i) a compound that increases expression of a CFTR in an epithelial cell; and/or

(ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and

(c) a physiologically acceptable carrier.

52. A pharmaceutical composition for treatment of cystic fibrosis, comprising:

(a) genistein;

(b) one or more of:

(i) a compound that increases expression of a CFTR in an epithelial cell; and/or

(ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and

(c) a physiologically acceptable carrier.

53. A pharmaceutical composition for treatment of cystic fibrosis, comprising:

- (a) quercetin;
- (b) one or more of:
 - (i) a compound that increases expression of a CFTR in an epithelial cell; and/or
 - (ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and
- (c) a physiologically acceptable carrier.

54. A pharmaceutical composition for treatment of cystic fibrosis, comprising:

- (a) apigenin;
- (b) one or more of:
 - (i) a compound that increases expression of a CFTR in an epithelial cell; and/or
 - (ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and
- (c) a physiologically acceptable carrier.

55. A pharmaceutical composition for treatment of cystic fibrosis, comprising:

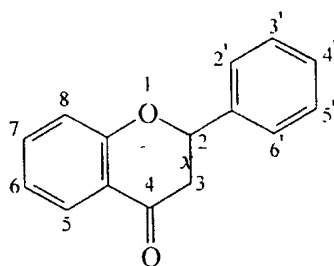
- (a) kaempferol;
- (b) one or more of:
 - (i) a compound that increases expression of a CFTR in an epithelial cell; and/or
 - (ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and
- (c) a physiologically acceptable carrier.

56. A pharmaceutical composition for treatment of cystic fibrosis.
comprising:

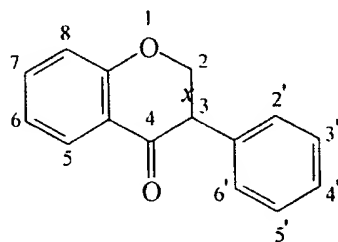
- (a) biochanin A;
- (b) one or more of:
 - (i) a compound that increases expression of a CFTR in an epithelial cell; and/or
 - (ii) a chemical chaperone that increases trafficking of a CFTR to a plasma membrane in an epithelial cell; and
- (c) a physiologically acceptable carrier.

57. A pharmaceutical composition for treatment of cystic fibrosis.
comprising:

- (a) a polyphenolic compound having the general formula:



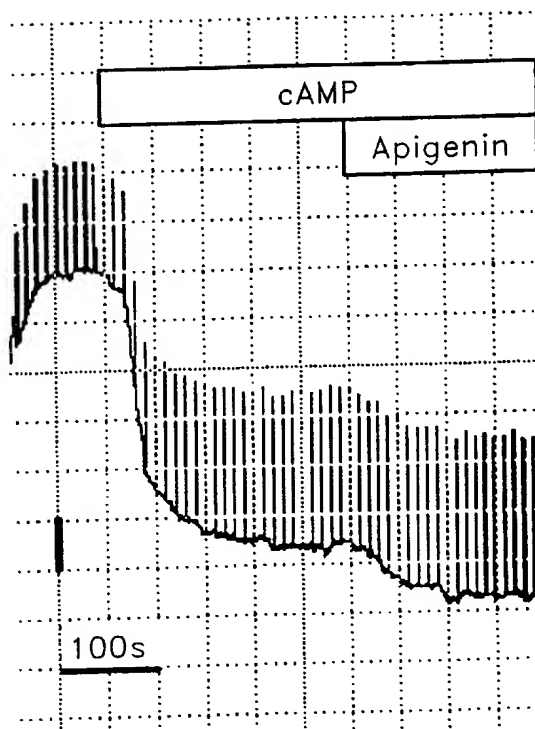
or



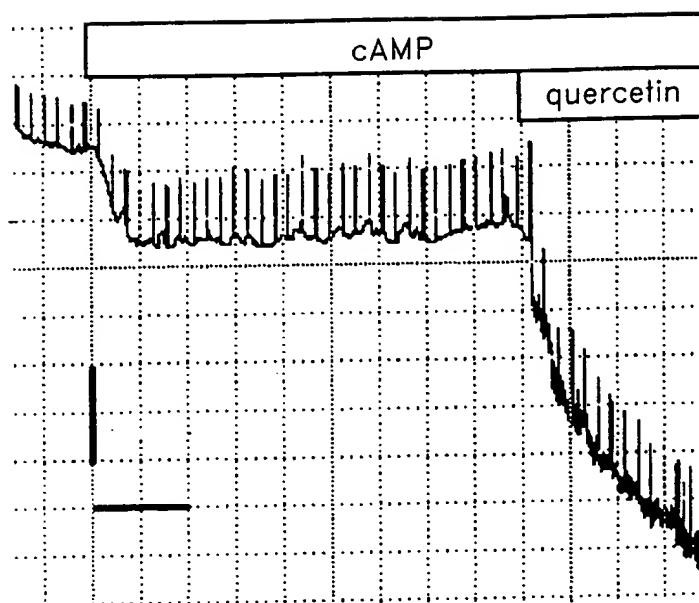
wherein carbon atoms at positions 2, 3, 5, 6, 7, 8, 2', 3', 4', 5' and 6' are bonded to a moiety independently selected from the group consisting of hydrogen atoms, hydroxyl groups and methoxyl groups, and wherein X is a single bond or a double bond; or a stereoisomer or glycoside derivative of any of the foregoing polyphenolic compounds;

- (b) a compound selected from the group consisting of resveratrol, ascorbic acid, ascorbate salts and dehydroascorbic acid; and
- (c) a physiologically acceptable carrier.

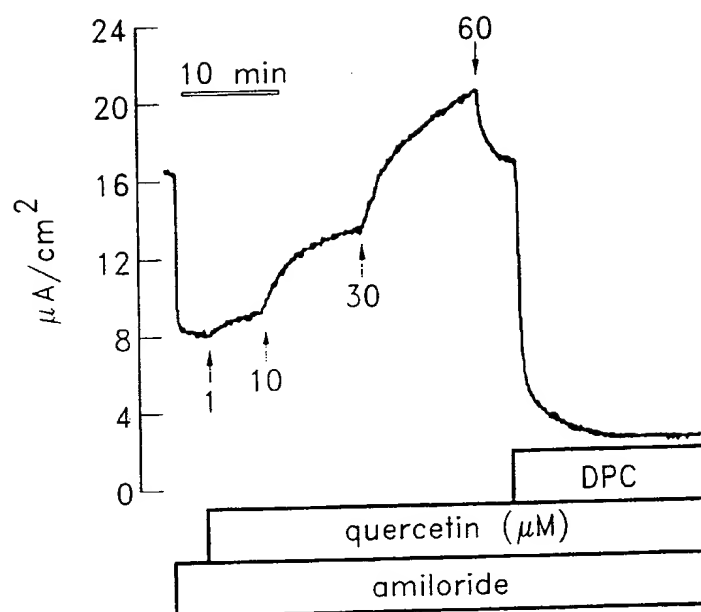
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*Fig. 1*

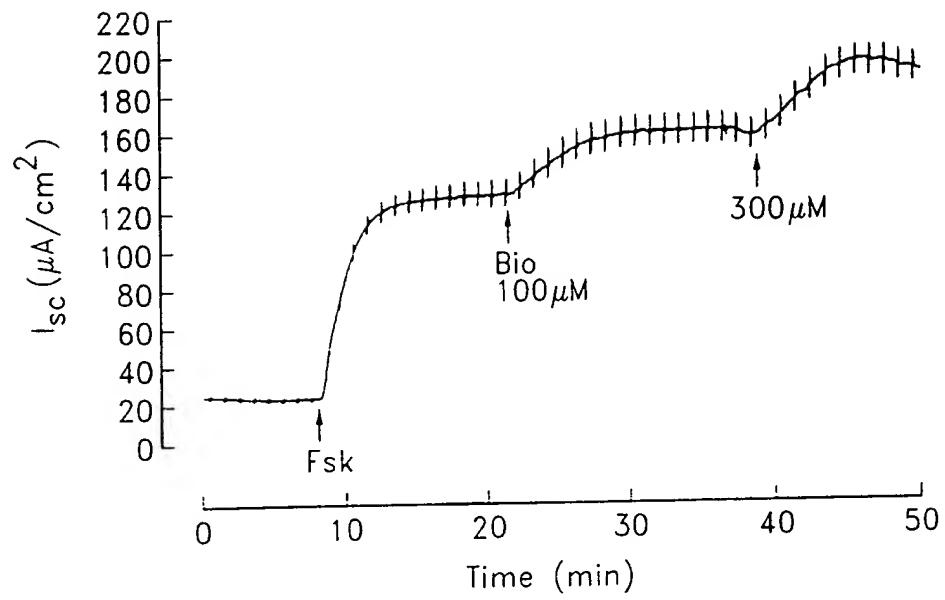
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*Fig. 2*

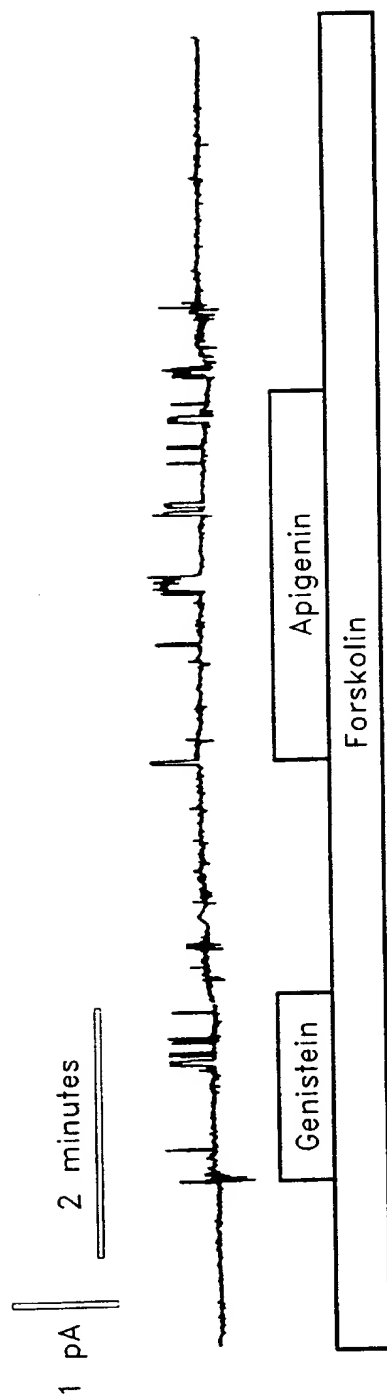
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*Fig. 3*

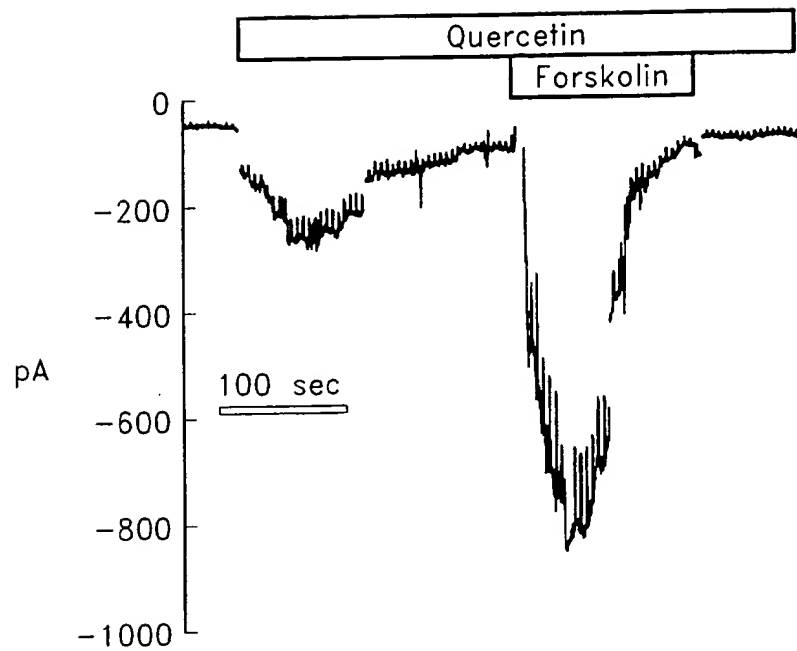
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*Fig. 4*

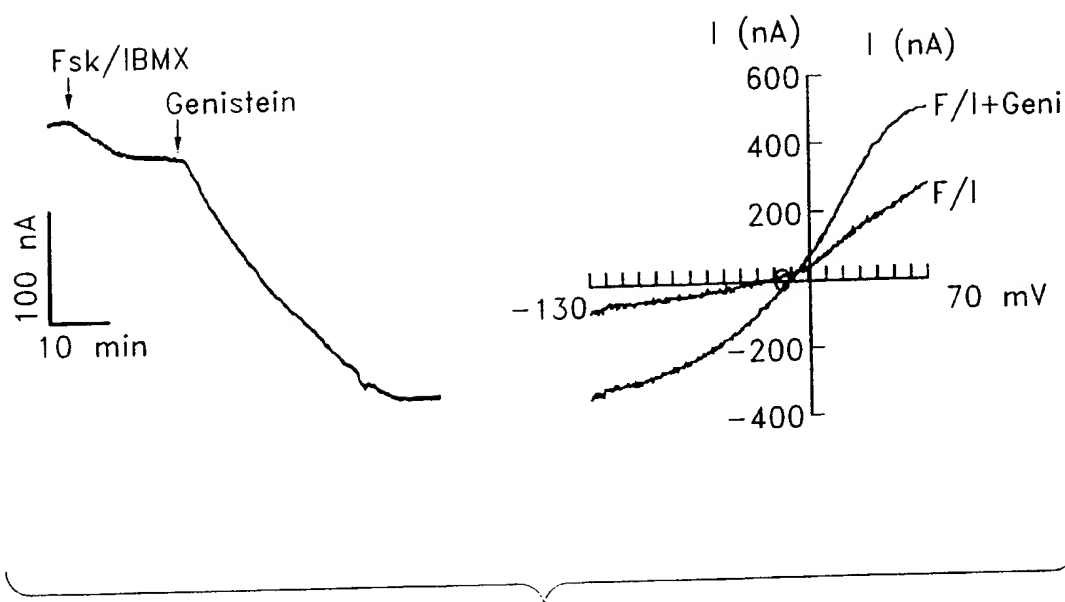
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*Fig. 5*

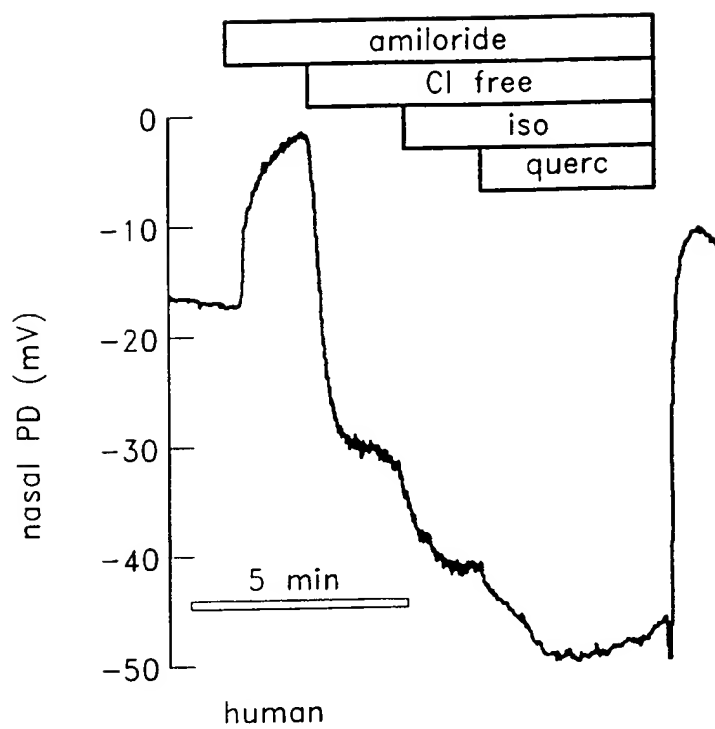
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*Fig. 6*

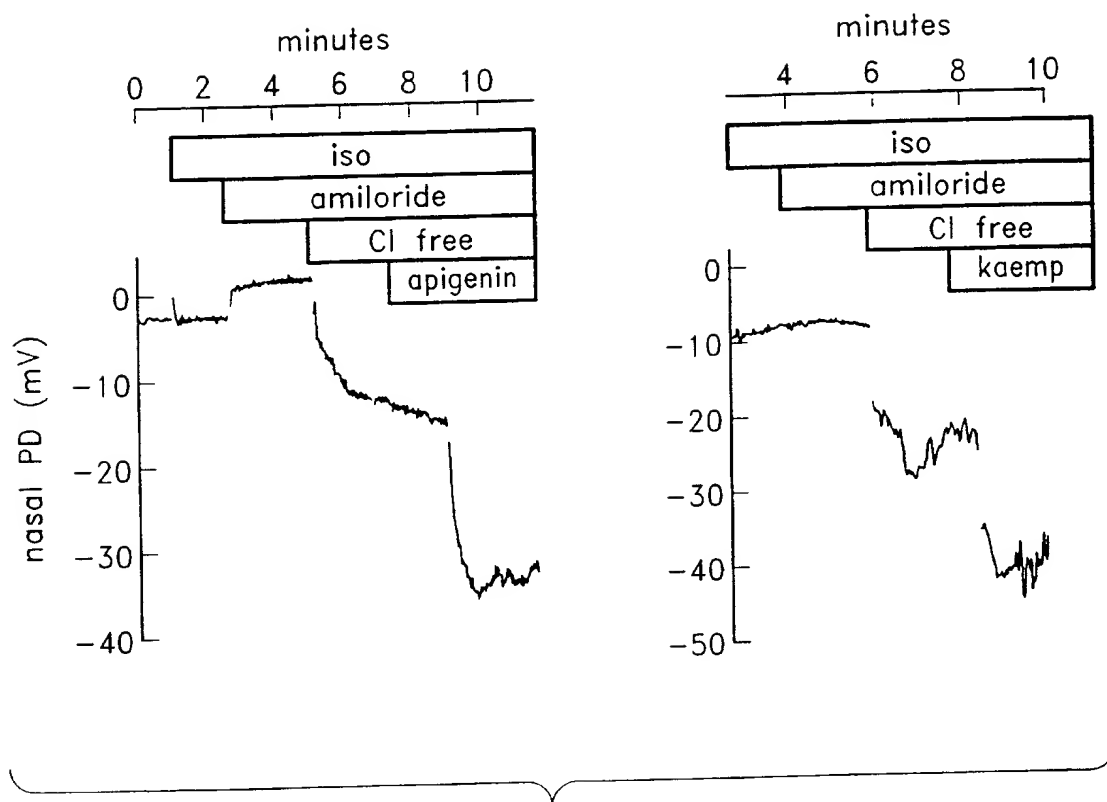
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*Fig. 7*

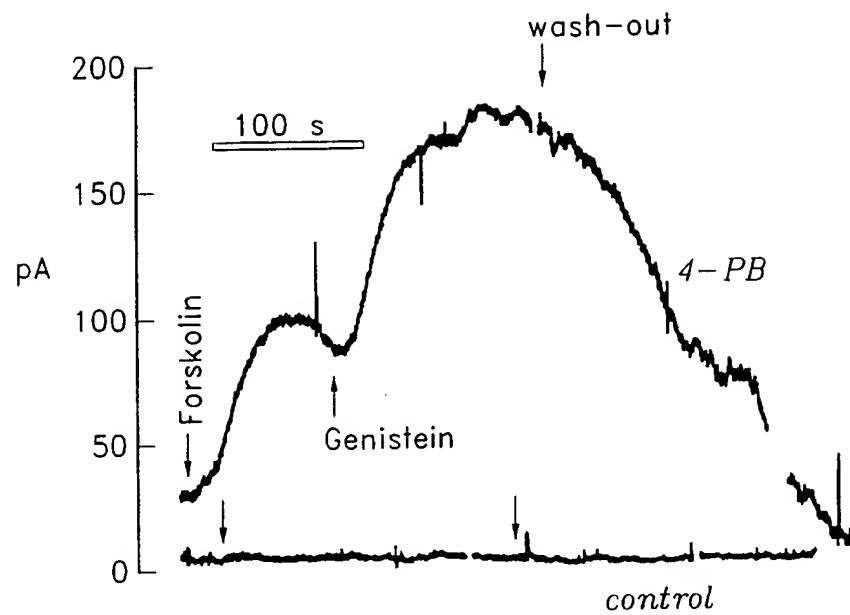
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*Fig. 8*

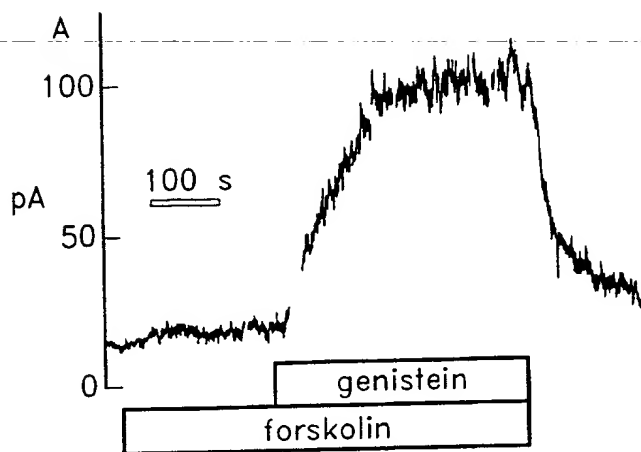
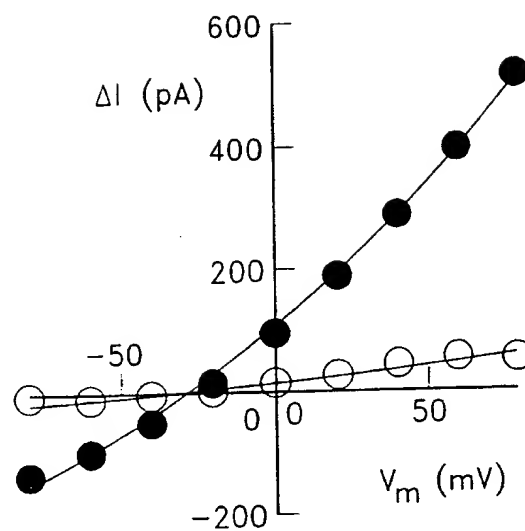
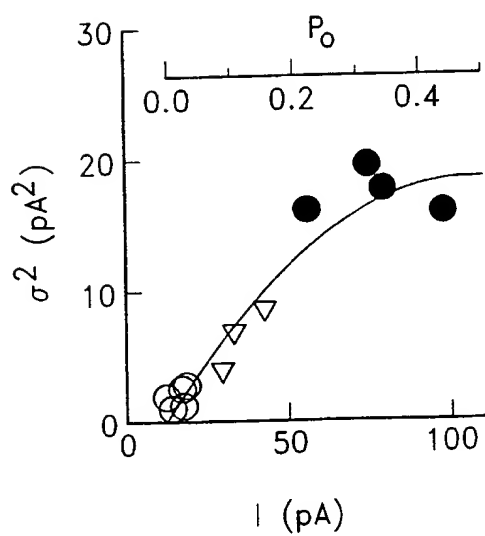
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*Fig. 9*

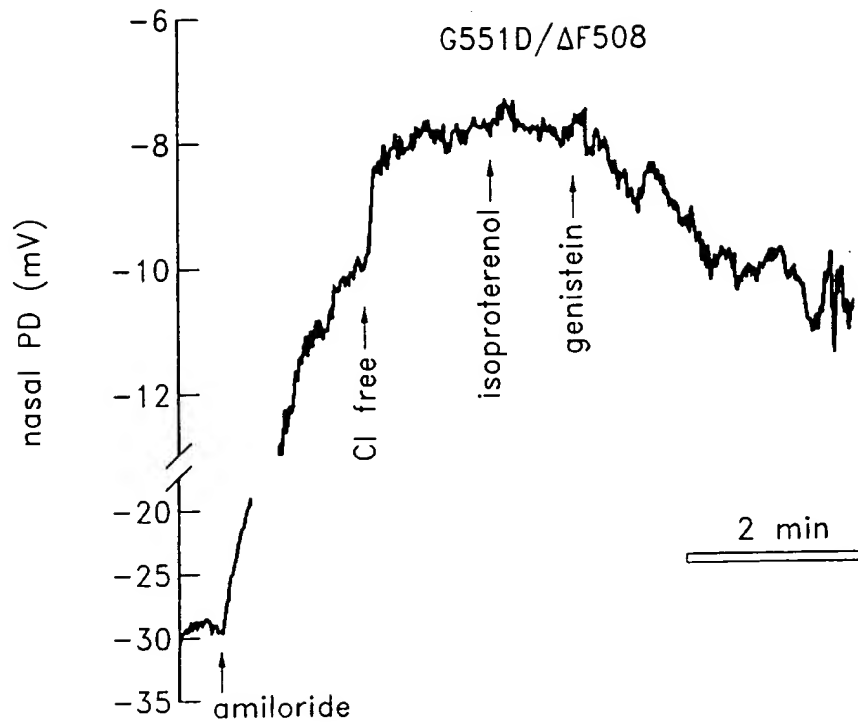
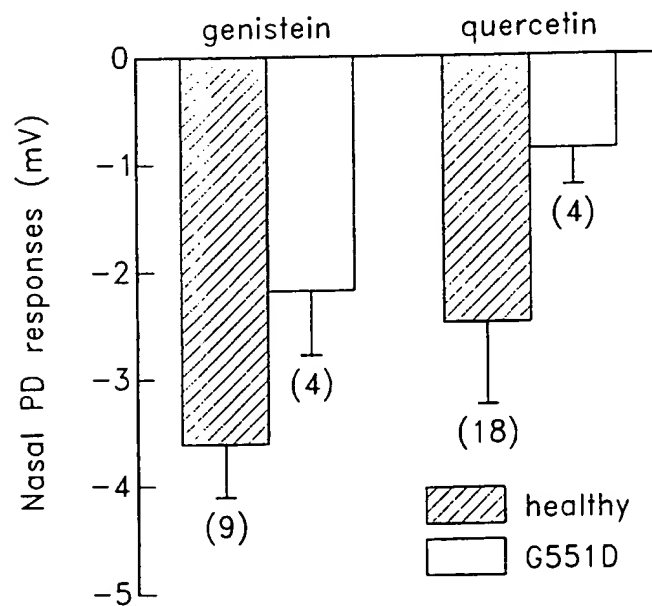
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*Fig. 10*

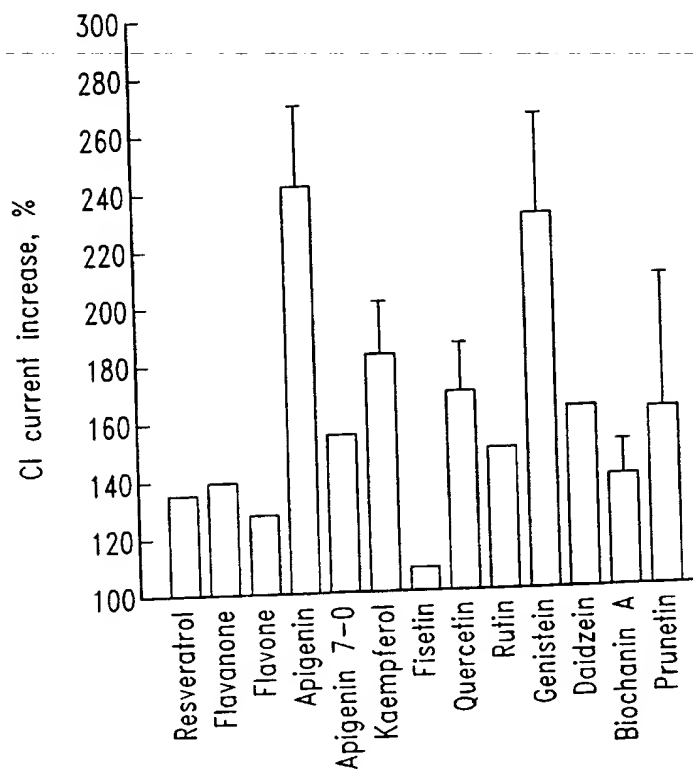
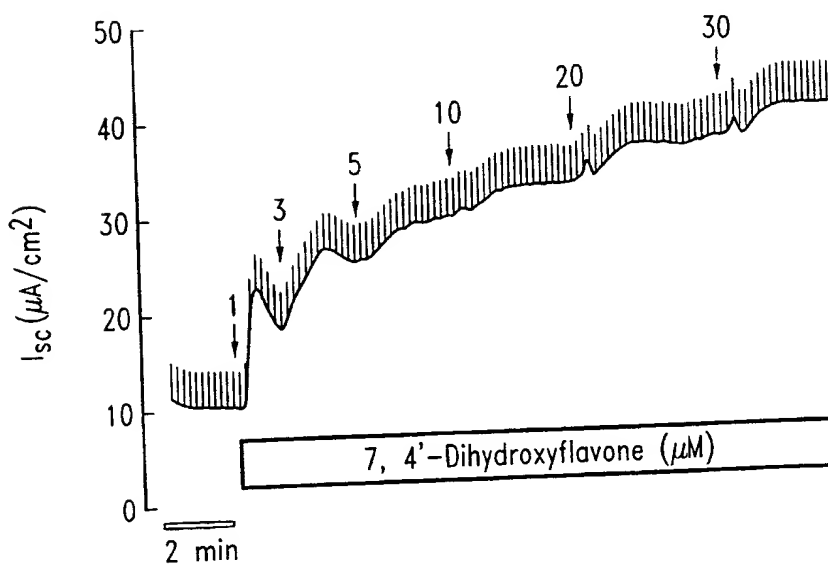
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*Fig. 11A**Fig. 11B**Fig. 11C*

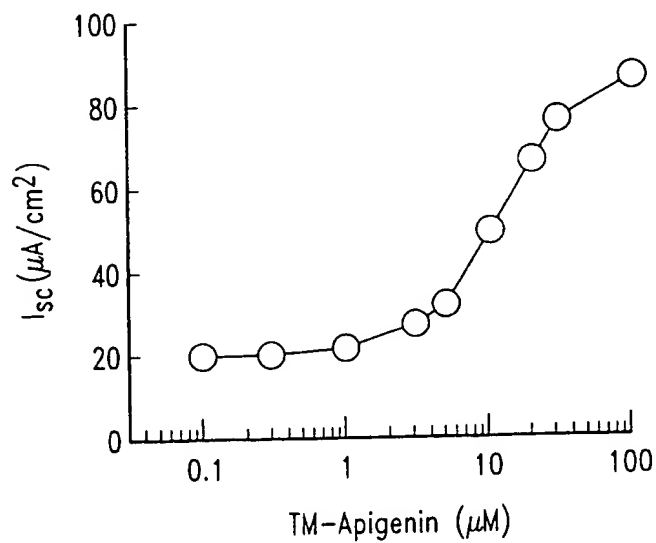
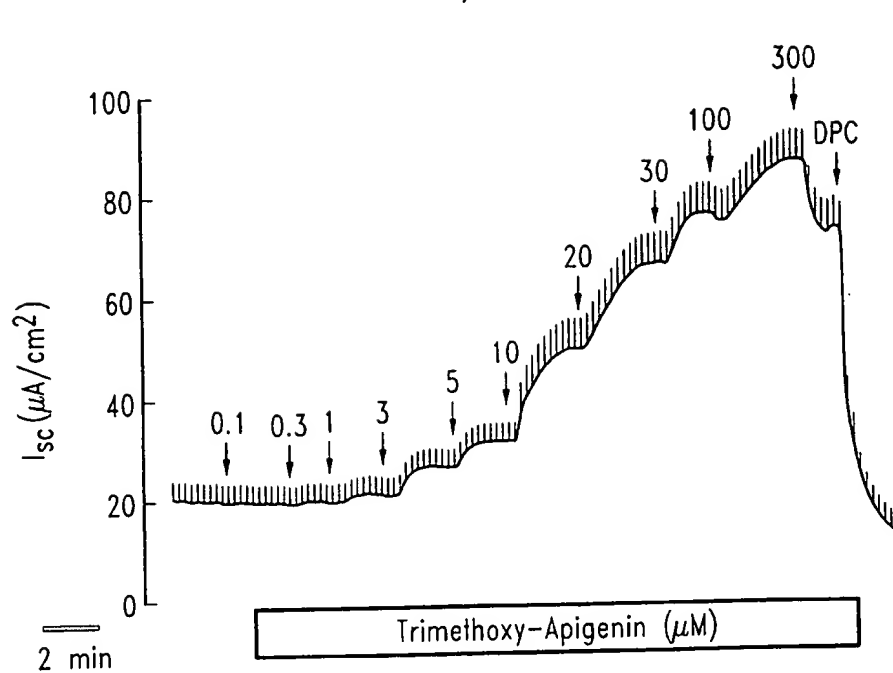
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*Fig. 12A**Fig. 12B*

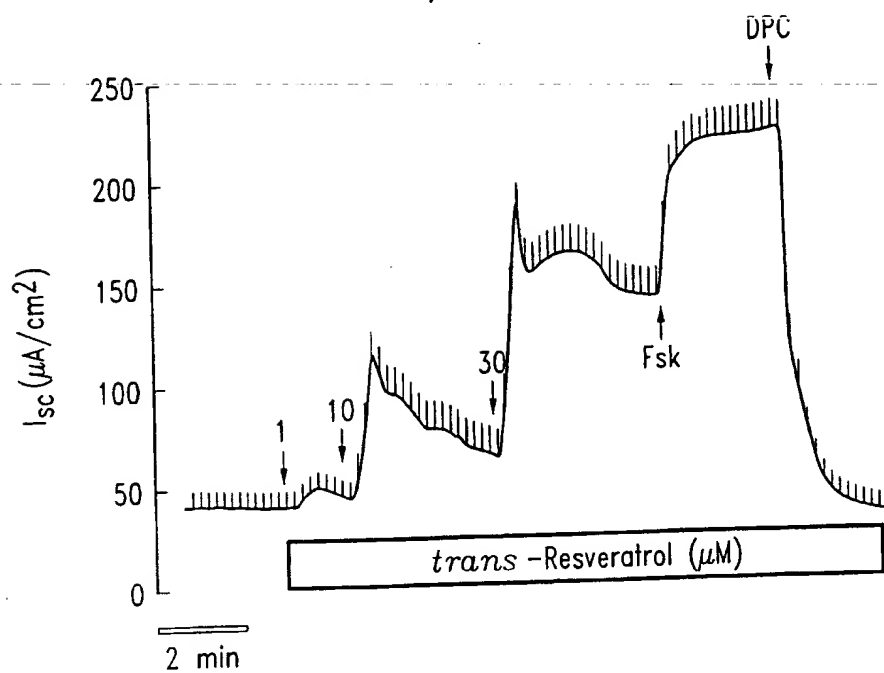
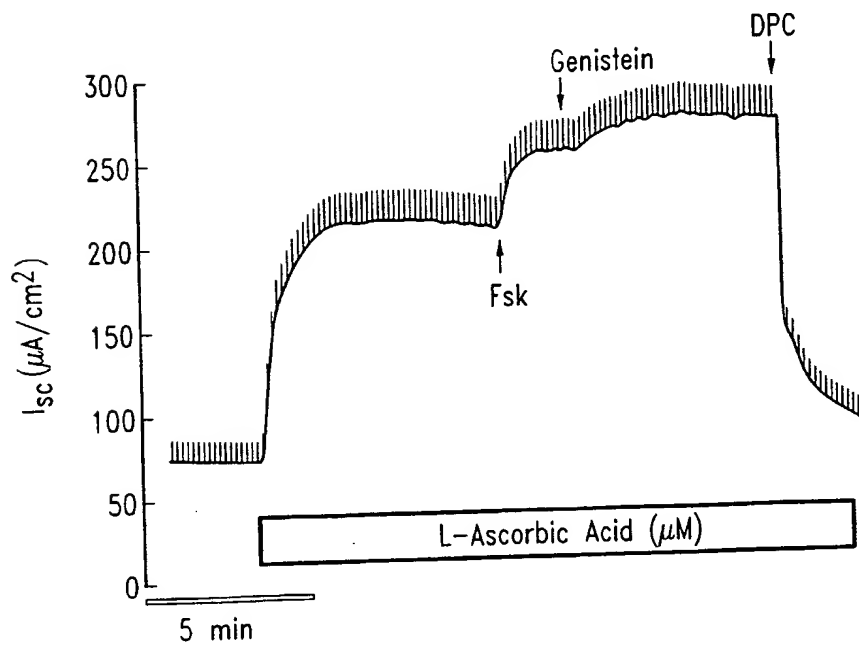
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*Fig. 13A**Fig. 13B*

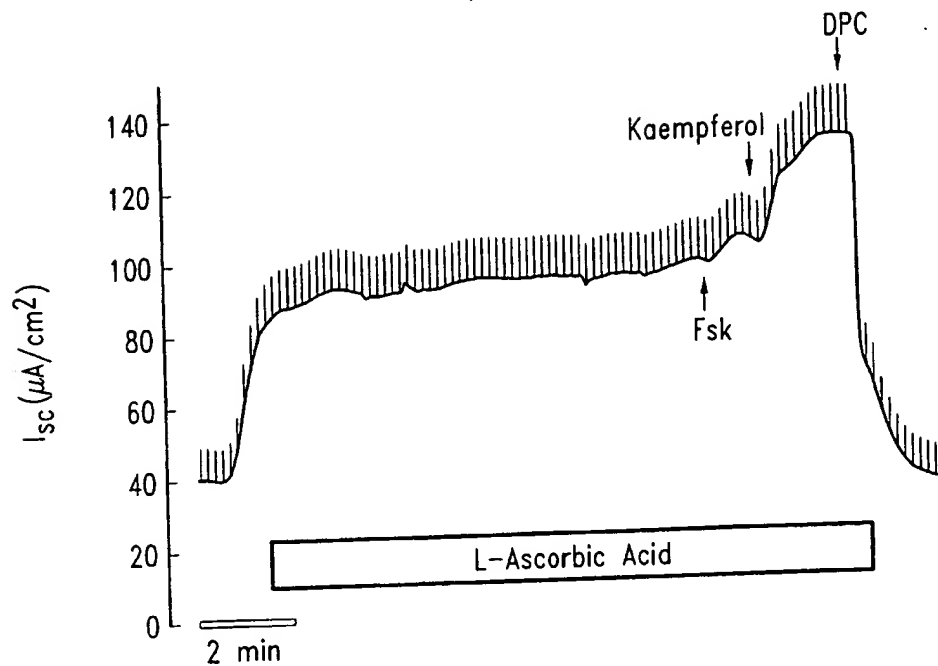
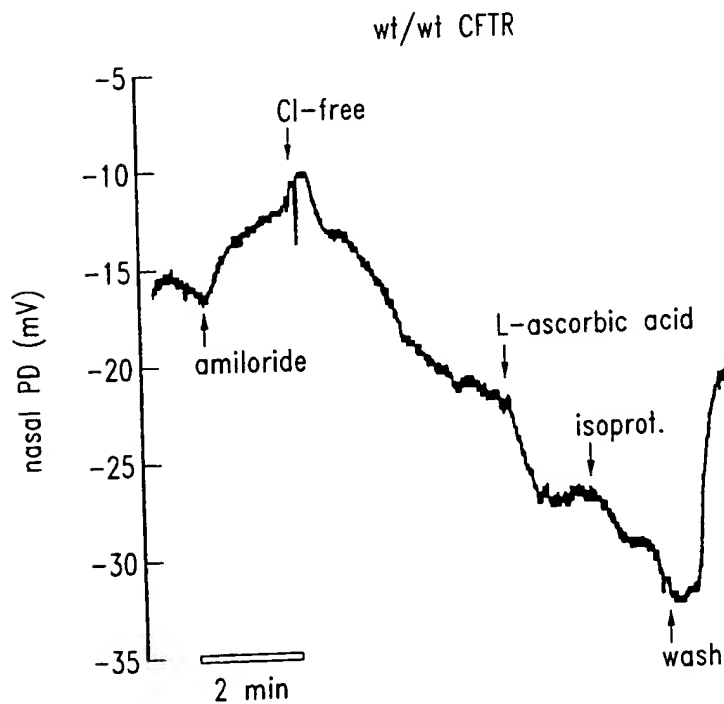
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*Fig. 13C*

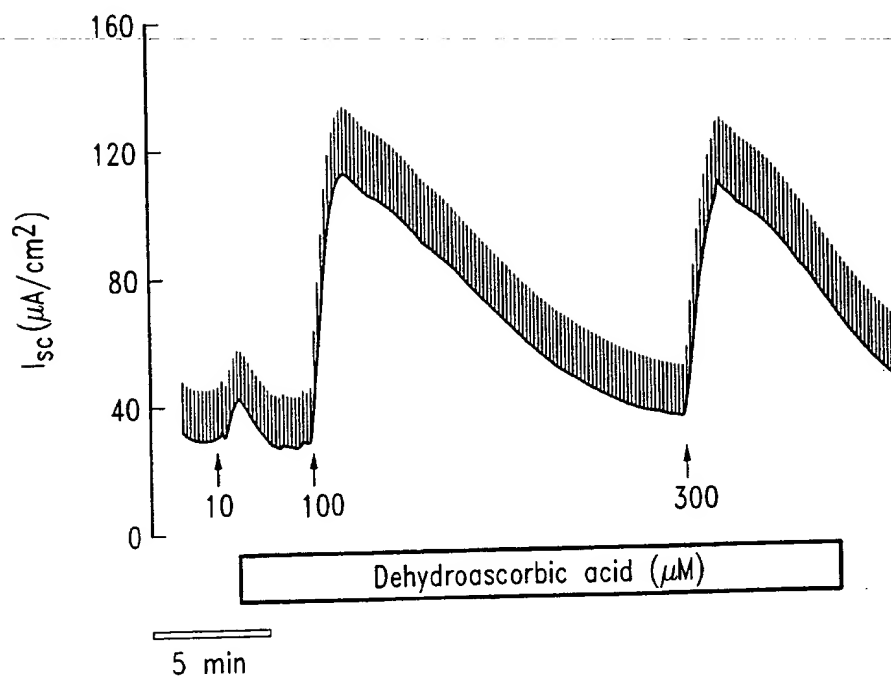
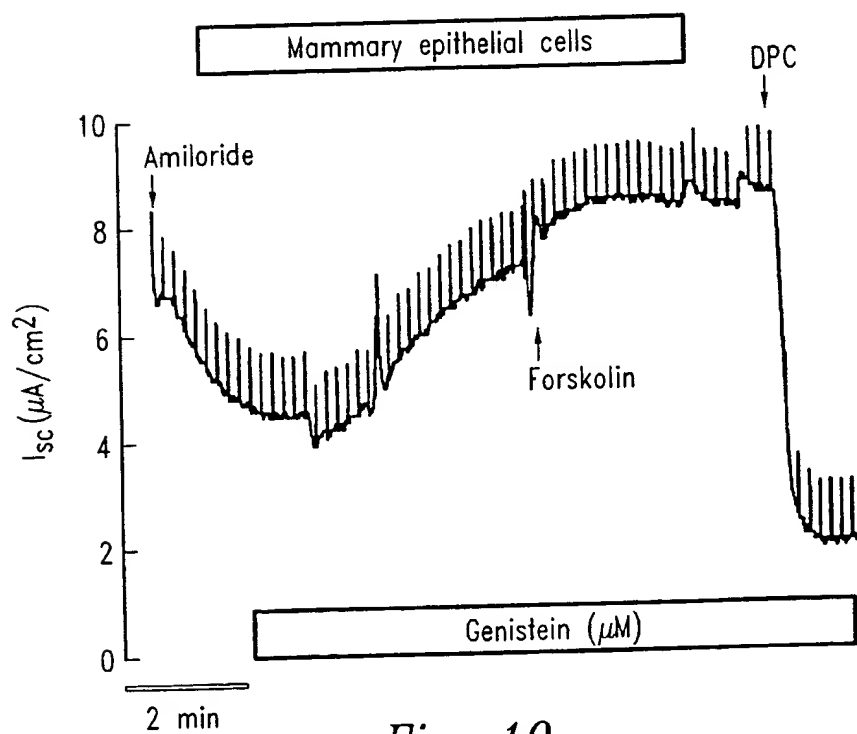
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*Fig. 14**Fig. 15*

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*Fig. 16**Fig. 17*

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*Fig. 18**Fig. 19*

SEQUENCE LISTING

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Illek, Beate

<120> COMPOSITIONS AND METHODS FOR CYSTIC FIBROSIS THERAPY

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His Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu Asn Glu Gly Ser Ser
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Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu Asp Lys Ile Ser

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Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu Asn Lys Phe Asp

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Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala Pro Leu Gln Val

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Val Tyr Lys Asp Ala Asp Leu Tyr Leu Leu Asp Ser Pro Phe Gly Tyr

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570

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Leu Met Ala Asn Lys Thr Arg Ile Leu Val Thr Ser Lys Met Glu His

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Leu Lys Lys Ala Asp Lys Ile Leu Ile Leu Asn Glu Gly Ser Ser Tyr

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Phe Tyr Gly Thr Phe Ser Glu Leu Gln Asn Leu Gln Pro Asp Phe Ser

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630

635

640

Ser Lys Leu Met Gly Cys Asp Ser Phe Asp Gln Phe Ser Ala Glu Arg

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Arg Asn Ser Ile Leu Thr Glu Thr Leu His Arg Phe Ser Leu Glu Gly

660

665

670

Asp Ala Pro Val Ser Trp Thr Glu Thr Lys Lys Gln Ser Phe Lys Gln

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680

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Thr Gly Glu Phe Gly Glu Lys Arg Lys Asn Ser Ile Leu Asn Pro Ile

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Val Ile Ser Thr Gly Pro Thr Leu Gln Ala Arg Arg Arg Gln Ser Val
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Leu Asn Leu Met Thr His Ser Val Asn Gln Gly Gln Asn Ile His Arg
770 775 780

Lys Thr Thr Ala Ser Thr Arg Lys Val Ser Leu Ala Pro Gln Ala Asn
785 790 795 800

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Phe Asp Asp Met Glu Ser Ile Pro Ala Val Thr Thr Trp Asn Thr Tyr
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Leu Arg Tyr Ile Thr Val His Lys Ser Leu Ile Phe Val Leu Ile Trp
850 855 860

Cys Leu Val Ile Phe Leu Ala Glu Val Ala Ala Ser Leu Val Val Leu
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1375

Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu Lys Gln Ala Phe Ala

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Asn Lys Glu Glu Arg Ser Ile Ala Ile Tyr Leu Gly Ile Gly Leu Cys
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Tyr Lys Lys Thr Leu Lys Leu Ser Ser Arg Val Leu Asp Lys Ile Ser
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Ile Gly Gln Leu Val Ser Leu Leu Ser Asn Asn Leu Asn Lys Phe Asp
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Glu Gly Leu Ala Leu Ala His Phe Val Trp Ile Ala Pro Leu Gln Val
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Ala Leu Leu Met Gly Leu Ile Trp Glu Leu Leu Gln Ala Ser Ala Phe
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Cys Gly Leu Gly Phe Leu Ile Val Leu Ala Leu Phe Gln Ala Gly Leu
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Gly Arg Met Met Met Lys Tyr Arg Asp Gln Arg Ala Gly Lys Ile Ser
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Glu Arg Leu Val Ile Thr Ser Glu Met Ile Glu Asn Ile Gln Ser Val
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Lys Ala Tyr Cys Trp Glu Glu Ala Met Glu Lys Met Ile Glu Asn Leu
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Tyr Leu Arg Tyr Ile Thr Val His Lys Ser Leu Ile Phe Val Leu Ile
850 855 860

Trp Cys Leu Val Ile Phe Leu Ala Glu Val Ala Ala Ser Leu Val Val
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Tyr Phe Glu Thr Leu Phe His Lys Ala Leu Asn Leu His Thr Ala Asn
1075 1080 1085

Trp Phe Leu Tyr Leu Ser Thr Leu Arg Trp Phe Gln Met Arg Ile Glu
1090 1095 1100

Met Ile Phe Val Ile Phe Phe Ile Ala Val Thr Phe Ile Ser Ile Leu
1105 1110 1115 1120

Thr Thr Gly Glu Gly Glu Gly Arg Val Gly Ile Ile Leu Thr Leu Ala
1125 1130 1135

Met Asn Ile Met Ser Thr Leu Gln Trp Ala Val Asn Ser Ser Ile Asp
1140 1145 1150

Val Asp Ser Leu Met Arg Ser Val Ser Arg Val Phe Lys Phe Ile Asp
1155 1160 1165

Met Pro Thr Glu Gly Lys Pro Thr Lys Ser Thr Lys Pro Tyr Lys Asn

1170 1175 1180
 Gly Gln Leu Ser Lys Val Met Ile Ile Glu Asn Ser His Val Lys Lys
 1185 1190 1195 1200
 Asp Asp Ile Trp Pro Ser Gly Gly Gln Met Thr Val Lys Asp Leu Thr
 1205 1210 1215
 Ala Lys Tyr Thr Glu Gly Gly Asn Ala Ile Leu Glu Asn Ile Ser Phe
 1220 1225 1230
 Ser Ile Ser Pro Gly Gln Arg Val Gly Leu Leu Gly Arg Thr Gly Ser
 1235 1240 1245
 Gly Lys Ser Thr Leu Leu Ser Ala Phe Leu Arg Leu Leu Asn Thr Glu
 1250 1255 1260
 Gly Glu Ile Gln Ile Asp Gly Val Ser Trp Asp Ser Ile Thr Leu Gln
 1265 1270 1275 1280
 Gln Trp Arg Lys Ala Phe Gly Val Ile Pro Gln Lys Val Phe Ile Phe
 1285 1290 1295
 Ser Gly Thr Phe Arg Lys Asn Leu Asp Pro Tyr Glu Gln Trp Ser Asp
 1300 1305 1310
 Gln Glu Ile Trp Lys Val Ala Asp Glu Val Gly Leu Arg Ser Val Ile
 1315 1320 1325
 Glu Gln Phe Pro Gly Lys Leu Asp Phe Val Leu Val Asp Gly Gly Cys
 1330 1335 1340
 Val Leu Ser His Gly His Lys Gln Leu Met Cys Leu Ala Arg Ser Val
 1345 1350 1355 1360
 Leu Ser Lys Ala Lys Ile Leu Leu Leu Asp Glu Pro Ser Ala His Leu
 1365 1370 1375

Asp Pro Val Thr Tyr Gln Ile Ile Arg Arg Thr Leu Lys Gln Ala Phe

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Ala Asp Cys Thr Val Ile Leu Cys Glu His Arg Ile Glu Ala Met Leu

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Glu Cys Gln Gln Phe Leu Val Ile Glu Glu Asn Lys Val Arg Gln Tyr

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Asp Ser Ile Gln Lys Leu Leu Asn Glu Arg Ser Leu Phe Arg Gln Ala

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Ile Ser Pro Ser Asp Arg Val Lys Leu Phe Pro His Arg Asn Ser Ser

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Lys Cys Lys Ser Lys Pro Gln Ile Ala Ala Leu Lys Glu Glu Thr Glu

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Glu Glu Val Gln Asp Thr Arg Leu

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/21887

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/35 A61K31/05 A61K31/375

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HWANG ET AL.: "Genistein potentiates wild-type and deltaF508-CFTR channel activity" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 273, no. 3 Part 1, September 1997, pages C988-C998, XP002093436 see page C997, column 2	1-3, 5-13, 24-26
Y	---	14, 16-23, 27-29, 51,52,57
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☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 February 1999

Date of mailing of the international search report

02/03/1999

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCOTT ET AL: "Ascorbic acid stimulates chloride transport in the amphibian cornea" INVESTIGATIVE OPHTHALMOLOGY, vol. 14, no. 10, October 1975, pages 763-766, XP002093437 see table 1	4,15
Y	---	21-23, 29,57
Y	SMITH: "Treatment of cystic fibrosis based on understanding CFTR" J. INHER. METAB. DIS., vol. 18, 1995, pages 508-516, XP002064542 see page 510 "Restoration of trafficking of mutant CFTR"	14, 16-20, 27,51,52
Y,P	--- RUBENSTEIN ET AL.: "In vitro pharmacologic restoration of CFTR-mediated chloride transport..." JOURNAL OF CLINICAL INVESTIGATION, vol. 100, no. 10, November 1997, pages 2457-2465, XP002076381 see page 2464 "Conclusions"	14, 16-19, 27,51,52
Y	--- RANDELL BROWN ET AL.: "Chemical chaperones correct the mutant phenotype of the deltaF508 cystic fibrosis..." CELL STRESS & CHAPERONES, vol. 1, no. 2, 1996, pages 117-125, XP002093438 see abstract see "Discussion"	18,20, 28,51,52
X	--- DATABASE WPI Week 8715 Derwent Publications Ltd., London, GB; AN 87-105816 XP002093439 & JP 62 053923 A (TEIJIN LTD.) , 9 March 1987 see abstract	54
X	--- DATABASE WPI Week 9342 Derwent Publications Ltd., London, GB; AN 93-330545 XP002093440 & JP 05 236910 A (NISSHIN FLOUR MILLING CO.), 17 September 1993 see abstract --- -/--	57

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/21887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE WPI Week 9434 Derwent Publications Ltd., London, GB; AN 94-277493 XP002093441 & RU 2 008 015 C (MAKSYUTINA) , 28 February 1994 see abstract</p>	57
X	<p>DATABASE WPI Week 9518 Derwent Publications Ltd., London, GB; AN 95-135875 XP002093442 & JP 07 059548 A (YAEGAKI HAKKO GIKEN KK) , 7 March 1995 see abstract</p>	57
A	<p>SHEPPARD ET AL.: "Mutations in CFTR associated with mild-disease-form chloride channels with altered pore properties" NATURE, vol. 362, 11 March 1993, pages 160-164, XP000612158 see the whole document</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/21887

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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